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14. ABSTRACT Our breast cancer research program initially focused on tumor suppressor BRCA1. In the past few years, we elucidated how BRCA1 is regulated and participates in the maintenance of genomic stability. Our studies demonstrated that loss of BRCA1 function leads to cell cycle checkpoint and repair defects and thus contributes to the development of familial breast cancer. Recently, we expanded our research program beyond BRCA1 and DNA damage responsive pathways. We studied several other signal transduction pathways, which are equally important for the maintenance of genomic stability and cell proliferation. These include Chfr and mitotic checkpoint regulation, and DBC1 (Deleted in Breast Cancer 1) and its role in the regulation of SIRT1. Together these studies revealed how the deregulation or disruption of these pathways would lead to breast cancer development. Moreover, we initiated several largescale studies, which we hope will provide potential targets for the development of anticancer therapy.					
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Introduction:

Genomic instability is the underlying mechanism for tumorigenesis, since it allows the accumulation of multiple genetic alternations, which are essential for the initiation of tumorigenesis. This has been clearly illustrated in familial breast cancer, since human genetic studies reveal that many genes involved in DNA damage response and DNA repair, including p53, BRCA1 (Breast cancer susceptibility gene 1) and BRCA2 (Breast cancer susceptibility gene 2), are frequently mutated and responsible for the development of hereditary breast and ovarian cancers. However, it remains unclear how genomic instability arises in sporadic breast cancers. The main focus of this proposal is to understand the signaling networks that ensure genomic integrity in humans. The short-term goal is to understand how deregulation or disruption of these networks may contribute to breast cancer development. The long-term objective is to build upon this knowledge and develop biomarkers and identify new targets for clinical applications. As a basic scientist devoted to breast cancer research, I am focusing my effort on elucidating the DNA damage response and the control of mitotic progression, two main pathways that help to maintain genomic integrity at DNA and chromosomal levels. We hope that the in-depth studies of these pathways and our attempt to develop new biomarkers and targets for therapeutic interventions will help eradicating breast cancer in the near future.

Body:

The Specific Aims are:

Specific Aim 1: Develop biomarkers for early detection of breast cancers.

The objective of this specific aim is to understand early genetic alternations that would eventually lead to the development of malignant breast cancers.

Based on our central hypothesis that genomic instability is the driving force of tumorigenesis, we developed several immunohistochemical (IHC) assays for the assessment of DNA damage checkpoint activation in breast cancer and/or early lesions. These include pATM [an activated form of ataxia telangiectasia mutated (ATM) kinase], pRPA [phosphorylated replication protein A (RPA) at Thr 21 site, which depends on the ATR (ATM and Rad3 related) kinase]. We also studied chromosomal instability, especially how several key mitotic regulators CHFR, Aurora A and PLK1 contribute to tumorigenesis. We established IHC assay for CHFR and demonstrated that CHFR is downregulated in some breast cancer samples. These results were summarized in 2006 annual report.

Although BRCA1 is frequently mutated in familial breast cancer, BRCA1 mutations are rare in sporadic breast cancer, raising the possibility that other components in the DNA damage responsive pathways and/or BRCA1-dependent pathways may be mutated or deregulated in sporadic cancers. We decided to further delineate the DNA damage pathways in which BRCA1 participates. As summarized in 2007, 2008 and 2009 annual reports, we identified an ubiquitin-dependent signaling transduction pathway, which

relies on an E3 ubiquitin ligase RNF8 (RING domain nuclear factor 8) and a protein complex that consists of RAP80, CCDC98/ABRAXAS, BRE/BRCC45, BRCC36, MERIT40/NBA1 for the recruitment of BRCA1 to sites of DNA damage (Feng et al., 2009; Huen et al., 2007; Kim et al., 2007a; Kim et al., 2007b). Several other groups also reported similar findings (Kolas et al., 2007; Liu et al., 2007; Mailand et al., 2007; Shao et al., 2009; Sobhian et al., 2007; Wang and Elledge, 2007; Wang et al., 2009; Wang et al., 2007; Yan et al., 2007). Moreover, we showed that this ubiquitin-dependent signaling pathway is also required for the recruitment of other DNA damage repair proteins such as PTIP/PA1 and RAD18 to DNA damage sites (Gong et al., 2009; Huang et al., 2009b), indicating that this pathway may play a general role in DNA damage response. These observations have promoted others to examine whether any of these components would be mutated in breast cancers. One mutation in RAP80 has been identified in a high risk breast cancer family (Nikkila et al., 2009), while other studies did not find any significant mutations in RAP80, CCDC98/Abraxas, or MERIT40/NBA1 (Akbari et al., 2009; Novak et al., 2009; Solyom et al., 2009). However, a more recent genome-wide association study suggests that MERIT40 may be involved in epithelial ovarian cancer development (Bolton et al., 2010). Together, these data support our hypothesis that some of the components involved in BRCA1-dependent pathways are mutated in cancer and may contribute to breast and other cancer development.

Besides the RNF8-dependent DNA damage signaling pathway discussed above, we also know that there is a H2AX/RNF8-independent pathway involved in the recruitment of BRCA1 and other DNA damage repair proteins to sites of DNA breaks (Celeste et al., 2003). As presented in 2009 annual report, we showed that this H2AX/RNF8-independent pathway requires the MRE11/RAD50/NBS1 (MRN) complex (Yuan and Chen, 2010). These data suggest that there are overlapping signaling pathways involved in the recruitment of many DNA damage repair proteins, which together enhance the overall efficiency of DNA repair.

Another interesting aspect of BRCA1 function is its involvement in homologous recombination (HR) repair. As presented in 2008 annual report, we believe that this function of BRCA1 is at least in part mediated by its physical interaction with the PALB2/BRCA2/RAD51 complex. In support of this hypothesis, we showed that several BRCA1 mutations identified in patients with family history of breast and ovarian cancers disrupted or reduced the interaction between BRCA1 and PALB2, and subsequently diminished the efficiency of HR repair (Sy et al., 2009b). Like BRCA1 and BRCA2, PALB2 is also mutated in breast cancer patients and in patients with Fanconi Anemia syndrome (Erkko et al., 2007; Foulkes et al., 2007; Rahman et al., 2007; Reid et al., 2007; Simpson, 2007; Tischkowitz et al., 2007; Walsh and King, 2007; Wang, 2007; Xia et al., 2007). These data strongly support the theory that HR defect is the main underlying mechanism for the development of familial breast cancer. Thus, we expanded our studies on HR repair.

As presented in 2009 annual report, we identified MRG15 as a new PALB2-associated protein and further delineated the roles of PALB2 in HR repair (Sy et al., 2009a; Sy et al., 2009c). We also studied the interaction between MRN complex and a DNA damage

repair protein CtIP and showed that the N-terminus of CtIP binds to MRN complex and dictates its function in HR (Yuan and Chen, 2009). In addition, we isolated two related Sensor Of Single-Strand DNA (SOSS) complexes and revealed that these complexes also work with MRN complex in HR (Huang et al., 2009a).

In 2010 annual report, we presented data on the discovery and analysis of FAN1 (Fanconi anemia associated nuclease 1), which associates with monoubiquitinated FANCI/FANCD2 and participates in cell survival following MMC treatment (Liu et al., 2010). In addition, we also reported the identification of two novel proteins, C9orf119 and C10orf78, as RAD51-associated proteins. C9orf119 and C10orf78 are human homologs of yeast Mei5/Swi5 complexes. We showed that this evolutionally conserved protein complex acts downstream of RPA, but is specifically required for RAD51 loading following DNA damage (Yuan and Chen, 2011). This manuscript is included in Appendix.

BRCA1 not only participates in DNA double-strand break (DSB) induced cell cycle checkpoint control and DNA repair, but also plays a role in replication stress-induced checkpoint control. Replication stress activates an ATR/Chk1 dependent pathway, which mainly acts in S/G2 phase of the cell cycle. The key regulator involved in ATR activation is a checkpoint protein TopBP1 (Topoisomerase II binding protein 1). As presented in 2010 annual report, we demonstrated that a specific interaction between TopBP1 and BACH1 is likely to be required for the extension of single-strand DNA regions and RPA loading following replication stress, which is pre-requisite for the subsequent activation of replication checkpoint (Gong et al., 2010). In addition, we found that TopBP1 also binds to MDC1 and this interaction plays an important role in Chk1 activation following replication stress (Wang et al., 2011). The manuscript describing TopBP1/MDC1 interaction is included in Appendix.

Moreover, an annealing helicase HARP (HepA-related protein, also called SMARCAL1--SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin subfamily a-like 1) is recruited to stalled replication forks via its association with RPA and functions to achieve a fine balance between checkpoint activation and stabilization of stalled replication forks (Yuan et al., 2009). In 2010 annual report, we showed that the annealing helicase activity of HARP is determined by its unique HARP motifs (Ghosal et al., 2011). Please see this manuscript in Appendix.

We are continuing to identify new components involved in DNA damage repair pathways that would act with BRCA1 and contribute to the maintenance of genomic stability and tumor suppression. Specifically, we have discovered another RAD51 binding protein.

FIGNL1 is a novel RAD51 binding protein

In the process of analyzing RAD51-containing protein complexes isolated from cells, we also uncovered another putative RAD51-associated protein, FIGNL1. FIGNL1 is a fidgetin (FIGN) like protein, which belongs to a subfamily of proteins containing

ATPases associated with diverse cellular activities (AAA) domain (Cox et al., 2000). We confirmed that FIGNL1 specifically associate with RAD51, but not RAD51 paralogs (**Figure 1A**). Moreover, using bacterially expressed and purified proteins, we showed that FIGNL1 binds directly to RAD51 (**Figure 1C**). This interaction requires a region of FIGNL1 that does not share any sequence homology with other proteins in the database (**Figure 1B and 1C**).

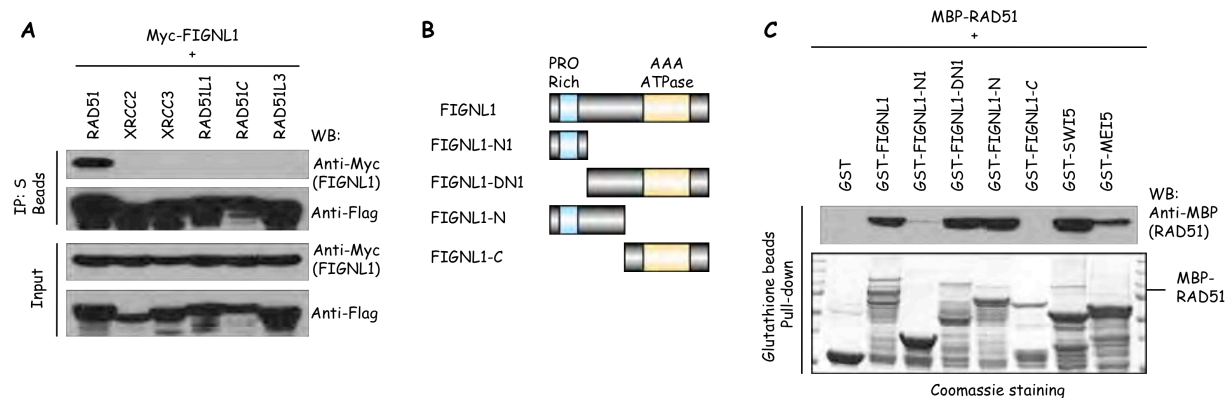


Figure 1. (A) Cells were transfected with constructs encoding Myc-tagged FIGNL1 along with constructs encoding SFB-tagged RAD51 or RAD51 paralogs. Co-precipitation experiments were conducted using S protein beads to pull down SFB tagged proteins and immunoblotting was conducted using antibodies as indicated. **(B)** Schematic diagrams of wild-type and mutant FIGNL1 used in this study. **(C)** GST pull down experiments confirmed a direct interaction between FIGNL1 and RAD51.

We are still exploring the functional significance of FIGNL1. We are generating FIGNL1 antibodies to confirm the interaction between endogenous FIGNL1 and RAD51. In addition, we will determine whether FIGNL1 participates in HR repair and whether it acts upstream or downstream of RAD51 in this process.

Besides DNA damage checkpoints, senescence is also a barrier to cancer development. We are interested in how senescence and/or aging process are normally regulated in humans and whether deregulation of this process would contribute to breast cancer development. Since protein deacetylase SIRT1 is evolutionary conserved and required for aging or longevity regulation from yeast to mice, we started from SIRT1 and examined how SIRT1 activity may be regulated *in vivo*. As summarized in 2007 annual report, we used a modified tandem affinity purification approach and identified DBC1 (deleted in breast cancer 1) as a major SIRT1-associated protein. Moreover, we demonstrated that DBC1 is a negative regulator of SIRT1 and participates in SIRT1 dependent stress responsive pathways (Kim et al., 2008). More recently, we identified Tip60 as a new SIRT1 substrate and demonstrated that SIRT1 regulates Tip60 autoacetylation and its function in apoptosis following DNA damage (Wang and Chen, 2010).

Specific Aim 2: Explore CHFR/Aurora pathway for breast cancer development and treatment.

Besides DNA damage responsive pathways, we also study mitotic progression

especially how the disruption of proper mitotic control would lead to chromosomal instability and tumorigenesis. We studied a mitotic checkpoint protein CHFR. We showed that as an E3 ligase, CHFR controls the expression of several key mitotic regulators including Aurora A and PLK1 and thus ensure chromosomal integrity during mitotic transitions (Yu et al., 2005). As introduced in our 2008 and 2009 annual reports, we also identified Kid/Kif22 and TOPK as new CHFR substrates. We showed that Chfr-mediated Kif22 downregulation is involved in early mitotic checkpoint control and the maintenance of chromosome stability (Maddika et al., 2009).

While CHFR is downregulated in a subset of breast cancers (Privette et al., 2007), Aurora A is often overexpressed in breast cancer. Indeed, Aurora kinase inhibitors including VX680 have been developed as potential anti-tumor agents. We were interested in determining the efficacy of Aurora A inhibitors and the mechanisms of potential resistance that may arise in tumors. However, as mentioned in 2007 annual report, we did not complete this study. This is because that in a project supported by NIH we generated Aurora A deficient mice and found surprisingly that Aurora A^{+/−} mice developed tumors at a higher frequency than wild-type littermates (Lu et al., 2008). These data raised the possibility that a partial inhibition of Aurora A function may promote tumorigenesis. Specific Aurora A inhibitors may have side effects of inducing secondary tumors. Because of these unexpected findings, we did not further pursue study on Aurora A kinase inhibitors. Instead, we collaborated with Dr. Taosheng Chen at St. Jude's children's hospital to screen for compounds that are selectively toxic to Chfr-deficient tumor cells. Unfortunately, as mentioned in 2008 annual report, we did not obtain any encouraging leads.

As presented in 2010 annual report, we are taking a cell biology approach to identify new components involved in mitotic regulations. The goal is to screen for the localization of ~16,000 full-length human ORFeome clones (Open Biosystems) in mitotic cells. We initiated this project last year. Until now, 3272 ORFs have been screened. During the course of this initial screening, we picked one candidate KIAA1383 for further analysis.

KIAA1383 is a previously uncharacterized protein. While this protein is conserved throughout evolution, it does not have clearly defined functional domains and does not share any extensive sequence homology with other proteins in the database. KIAA1383 has unique localization in mitotic cells (**Figure 2A**). During metaphase, it displays prominent staining at spindle poles and also on mitotic spindle, whereas it retains in intercellular microtubule bundle during telophase (**Figure 2A**). These data suggest that KIAA1383 is an integral component of mitotic apparatus.

To determine whether KIAA1383 is required for mitotic controls, siRNAs against KIAA1383 was synthesized and efficient knockdown of KIAA1383 was confirmed by immunoblotting (**Figure 2B**). In control cells, bipolar spindles were formed and the chromosomes were properly aligned at the spindle equator (**Figure 2C**). Upon KIAA1383 downregulation, two common types of defective mitotic apparatus (splitting

spindles and multipolar spindles) became apparent (**Figure 2C**), suggesting that KIAA1383 plays essential roles in chromosome segregation.

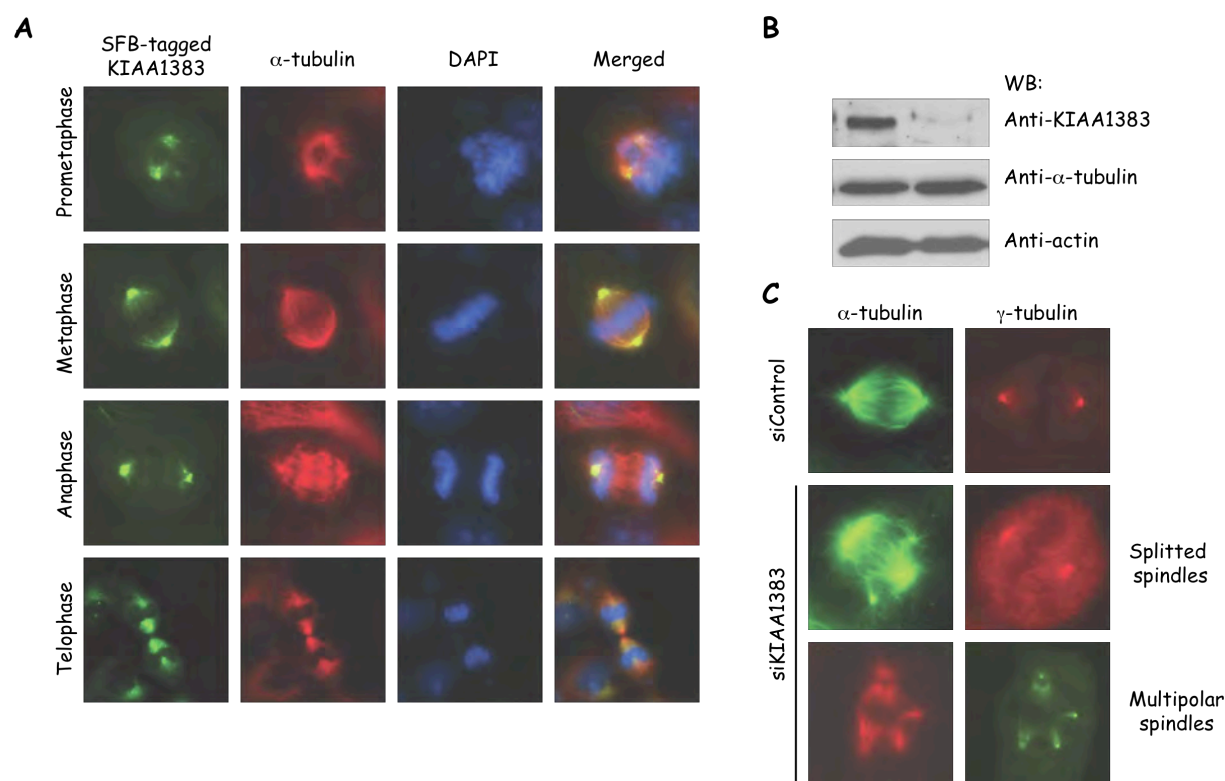


Figure 2. (A) Cell cycle–dependent distribution of KIAA1383. Cell line stably expressing SFB-tagged KIAA1383 was subjected to immunostaining using anti-Flag for KIAA1383, anti-α-tubulin for microtubules and DAPI for DNA. **(B, C)** Cells depleted of KIAA1383 exhibit mitotic defects. Cells were transfected with control or KIAA1383 siRNA for 96 hours. Downregulation of KIAA1383 was confirmed by Western blotting using antibodies as indicated (B). Cells transfected with control or KIAA1383 specific siRNAs were also fixed and immunostained with anti-α-tubulin for microtubules and anti-γ-tubulin for spindle poles (C).

Specific Aim 3: Identify novel druggable targets for the development of anti-cancer agents.

We are not only interested in achieving in-depth understanding of breast cancer etiology, but also would like to use the information for the development of anti-cancer therapy.

Because deficiency in DNA repair makes tumor cells more sensitive to radiation and other chemotherapeutic agents, we would like to develop compounds that inhibit BRCA1 functions and explore whether these compounds could increase the efficacy of existing chemotherapy and/or radiation therapy. We collaborated with Dr. Wei Wang at the University of New Mexico and Dr. Amar Natarajan at University of Texas Medical Branch Galveston to develop small compounds that would specifically disrupt the interaction between BRCA1 BRCT domains and phospho-proteins. We anticipated that such compounds would abolish BRCA1-dependent DNA damage checkpoint and repair functions. As summarized in 2006, 2007 and 2008 annual reports, we were able to

obtain some compounds that can reduce or abolish the interaction between BRCA1 and phospho-proteins *in vitro*. Unfortunately, further modifications of these compounds did not improve the efficacy of these compounds.

We did not succeed in these attempts to develop small molecules as potential anti-cancer agents. As a basic scientist, we may be able to contribute more in this arena by providing new targets for drug development. With this as a long-term goal, we want to purify epitope-tagged enzymes (e.g. protein kinases, phosphatases, E3 ubiquitin ligases, deubiquitinating enzymes, protein acetylases, deacetylases and others) from human cells for *in vivo* and *in vitro* studies. These reagents not only help us to understand the physiological functions of these enzymes, but will also provide essential tools for the validation of any specific inhibitors that may be developed in the future.

During the purification of these enzymes or enzyme complexes, we made several interesting discoveries. As presented in 2008 annual report, we found that Dual-specificity tyrosine (Y) - phosphorylation regulated kinase 2 (DYRK2) has an unexpected role as a component of an E3-ubiquitin ligase complex. DYRK2 is required for the formation of this E3 ligase complex as well as for the subsequent phosphorylation, ubiquitination and degradation of its substrates (Maddika and Chen, 2009). As presented in 2009 and 2010 annual reports, we found that the JAMM domain-containing deubiquitinating enzyme BRCC36 exist in two different complexes *in vivo* (Feng et al., 2010). One is the nuclear complex that contains RAP80, CCDC98/Abraxas, BRCC45/BRE and MERIT40/NBA1 (Feng et al., 2009; Shao et al., 2009; Wang et al., 2009). The other is a cytoplasmic complex contains BRCC45/BRE, MERIT40/NBA1 and a new component KIAA0157, which shares significant similarity with CCDC98/Abraxas. The major difference between CCDC98 and KIAA0157 is that KIAA0157 lacks the pSXXF motif at its very C-terminus, which is the motif that mediates the interaction between CCDC98 and BRCA1 (Kim et al., 2007b; Liu et al., 2007; Wang et al., 2007). Our subsequent studies suggest that these two BRCC36-containing complexes are regulated differently and may have distinct functions in the cell (Feng et al., 2010).

Training potential for the PI:

The most important lesson I learned during the course of this award is that our basic research needs to focus on solving clinical questions. While we are and will continue to conduct mechanism-based studies to understand the roles of genomic instability in breast cancer development, this training award gave me an opportunity to initiate several collaborative projects with clinicians, chemists and experts on high-throughput screening. Moreover, we realized that breast cancer is a complex disease and a comprehensive understanding of the landscape in breast cancer is the key for developing useful agents that would have meaningful clinical impact. Thus, we have expanded our research from the studies of individual proteins to intermediate-to-large scale studies, attempting to understand the interplays among different cellular pathways. I am confident that these large-scale studies will be fruitful and provide new targets for the development of anti-cancer treatment.

This award also gives me the greatest flexibility to train young scientists and allow them to develop their own careers in breast cancer research. Over the five-year period, this grant has provided research support for the following trainees: Zhenkou Lou, Xiaochun Yu, Zheng Fu, Hongtae Kim, Ja-Eun Kim, Liming Wu, Reddy Maddika, Michael Huen, Shirley Sy, Jun Huang, Zihua Gong, Jingsong Yuan, Lin Feng, Justin Leung and Jiadong Wang. Some of them have left my laboratory and established their own research teams in the United States, Korea, Hong Kong, India and China. Many of them are continuing breast cancer research, with topics ranging from DNA damage response, mitotic regulation, SIRT1 function to cell survival and proliferation. This diversity provides them opportunities to start up their own research program and contribute to many aspects of breast cancer research.

Key Research Accomplishments:

- Identified key components and pathways involved in the regulation of BRCA1 following DNA damage.
- Elucidated the mechanisms underlying replication checkpoint control and the prevention of replication fork collapsing and genomic instability in S phase cells.
- Isolated and studied several new proteins and protein complexes, which work with BRCA1 and RAD51 and participate in homologous recombination repair.
- Demonstrated that DBC1 is a key negative regulator of SIRT1.
- Revealed the roles of CHFR and other new components in the regulation of mitotic transitions and the maintenance of chromosomal stability.
- Uncovered novel functions of protein kinases and deubiquitinating enzymes in cell cycle control and DNA damage response.

Reportable Outcomes:

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Patents and Licenses: None

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Animal models and databases: None

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Employment or Research opportunities applied for: None

Conclusions:

Our research focused on the regulation of genomic stability and DNA damage responsive pathways. We discovered a new DNA damage-induced signaling pathway that regulates BRCA1 localization and functions. We identified a novel partner and regulator of SIRT1 as DBC1 (deleted in breast cancer 1) that may be involved in breast cancer development and aging. We established the role of tumor suppressor CHFR in the control of key mitotic kinases PLK1, Aurora A, and chromosomal stability. More recently, we focused our studies on the mechanisms of DNA repair, especially DNA double-strand break (DSB) repair and interstrand crosslink (ICL) repair. These are the two most lethal types of DNA lesions. Many agents that induce these lesions are routinely used for the treatment of breast cancers, indicating that breast cancers likely suffer from mutations or deregulation in components involved in the repair of these lesions. Our studies indicated that several overlapping pathways are involved in the repair of these DNA lesions. Further study of the coordination of these repair pathways may provide opportunities to combine current chemotherapeutic agents to improve the treatment for breast cancer patients.

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Appendices:

Manuscript 1: Yuan J, **Chen J**. The Role of the Human SWI5-MEI5 Complex in Homologous Recombination Repair. J Biol Chem. 286(11):9888-93, 2011.

Manuscript 2: Wang J, Gong Z, **Chen J**. MDC1 collaborates with TopBP1 in DNA replication checkpoint control. J Cell Biol. 193(2):267-73, 2011.

Manuscript 3: Ghosal G, Yuan J, **Chen J**. The HARP domain dictates the annealing helicase activity of HARP/SMARCAL1. EMBO Rep. 12(6):574-80, 2011.

The Role of the Human SWI5-MEI5 Complex in Homologous Recombination Repair^{*[5]}

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The Swi5-Mei5 complex and its homologues are involved in specialized recombination pathways in budding and fission yeasts. Although the fission yeast homologue Swi5-Sfr1 is critical for homologous recombination repair, the budding yeast counterpart Sae3-Mei5 is meiosis-specific, interacts with Dmc1, and promotes assembly of Dmc1 on meiotic chromosomes. Here, we identify and characterize the human SWI5-MEI5 (C9orf119-C10orf78) complex. We showed that SWI5 and MEI5 form a stable complex *in vitro* and *in vivo*. The C-terminal Swi5 domain of SWI5 and the middle coiled-coil region of MEI5 dictate this conserved interaction. In addition, SWI5-MEI5 directly interacts with RAD51 *in vitro*. Depletion of SWI5 or MEI5 in human cells causes defects in homologous recombination repair. Finally, SWI5- or MEI5-depleted cells display enhanced sensitivity to ionizing radiation, consistent with the role of this complex in HR repair. Our results suggest that human SWI5-MEI5 has an evolutionarily conserved function in homologous recombination repair.

The human genome is continuously challenged by all kinds of genotoxic stress, such as ultraviolet light, ionizing radiation, and endogenous processes, including recombination during normal immunological response and at stalled replication forks (1–3). Severe DNA lesions, such as double-strand breaks (DSBs)² and DNA cross-links, have to be appropriately repaired for cell survival. Inefficient or inaccurate repair of these lesions often lead to genomic instability and ultimately initiate cancer development (4, 5). DSBs are repaired mainly via two parallel repair pathways: the nonhomologous end-joining pathway and homologous recombination (HR) pathway. HR is particularly important for the repair of DSBs due to its ability to restore the genetic information, whereas repair via nonhomologous end-joining may potentially lead to deletions and mutations.

The central component in the HR pathway is RAD51, which is the major recombinase in mitotic cells and also plays a critical role during meiosis. RAD51 is the human homologue of *Escherichia coli* RecA. It ensures high fidelity DNA repair by facili-

tating strand exchange between homologous DNA segments (6, 7). BRCA2 is another key protein in HR, as it mediates the loading of RAD51 onto single-stranded DNA and stabilizing RAD51 filaments (8–10). BRCA2 is encoded by a tumor suppressor gene that, when mutated, greatly elevates risks for breast and ovarian cancer. Recently, another tumor suppressor PALB2 (partner and localizer of BRCA2) was also found to associate with BRCA2 and be required for the loading of the BRCA2-RAD51 repair complex onto DNA (11). PALB2 also serves as the molecular scaffold to link BRCA2 with the BRCA1 tumor suppressor (12, 13). In addition to BRCA2/PALB2, other important HR mediators are the five RAD51 paralogues (RAD51B, RAD51C, RAD51D, XRCC2, and XRCC3), which are required for the assembly of DNA damage-induced RAD51 foci, and cell lines with defects in any of these RAD51 paralogues are defective in HR (14–16). Given the importance of HR in the maintenance of genomic stability, it is not surprising that germ line mutations in many of the HR repair components, such as BRCA1, BRCA2, and PALB2, are associated with various human genetic disorders and cancers. Two recent studies identified biallelic mutations in RAD51C, which lead to Fanconi anemia-like disorder, and a monoallelic mutation in RAD51C that is associated with increased risk of breast and ovarian cancer (17, 18).

Studies in budding and fission yeasts have identified another protein complex Swi5-Mei5, which has an important role in HR (19–24). In budding yeast, the complex is named Sae3-Mei5. The Sae3-Mei5 complex is meiosis-specific, interacts with Dmc1, the meiosis-specific RecA homologue, and promotes the assembly of Dmc1 on meiotic chromosomes (20, 21, 24). In fission yeast, the Sae3 homologue is Swi5. There are two Swi5-containing complexes in fission yeast, Swi5-Sfr1 and Swi5-Swi2, with Sfr1 and Swi2 as budding yeast Mei5 homologues. Although Swi5-Sfr1 complex participates in an Rhp51 (the fission yeast RAD51 homologue)-dependent HR pathway (22), the Swi5-Swi2 complex is required for mating type switching (22, 25).

Although these studies suggest that Swi5-Mei5 is a conserved protein complex involved in HR, the human counterparts of these proteins have never been identified and characterized. In this study, we reported the discovery of the human SWI5-MEI5 complex. We showed that human SWI5 and MEI5 form a stable complex. SWI5-MEI5 directly interacts with RAD51 and plays a critical role in HR repair.

EXPERIMENTAL PROCEDURES

Antibodies—The antibody against RAD51 was described previously (12, 26). Anti-RPA2 antibody was obtained from

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² The abbreviations used are: DSB, double-strand break; HR, homologous recombination; MBP, maltose binding protein; IR, ionizing radiation; RPA, replication protein A.

Abcam. The anti-Myc antibody was obtained from Santa Cruz Biotechnology. Anti-FLAG (M2) were obtained from Sigma. Anti-maltose binding protein (MBP) antibody was raised by immunizing rabbits with purified full-length MBP protein. Antisera were affinity-purified using AminoLink plus Immobilization and purification kit (Pierce).

Cell Culture, Transfection, and siRNAs—U2OS and 293T cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin. Plasmid transfection was performed using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. The sequence for RAD51 siRNA was described previously (26, 27). ON-TARGETplus siRNA Sets for SWI5 (C9orf119) and MEI5 (C10orf78) were purchased from Dharmacon. The sequences of human SWI5 siRNAs were as follows: 18, CUGAAAUGUCGAGUGAUUU; 19, GAACCAAGACUUACCCGAAUU; 20, AGAGUUGUAUCCAGAGUUUU; and 21, GUUCGUAUCUGAAGGCUACUU. The sequences of human MEI5 siRNAs were: 9, AUACAAUA-GUCCCCGAAUU; 10, AAACAAAGAUUAAACGCU-GUU; 11, ACUAUGGGUUGAUGAUAAUU; and 12, CUGAUAGUCUAGCAGGUAUU. The siRNA transfection was performed using Oligofectamine (Invitrogen) following the manufacturer's instructions. Transfection was repeated once with an interval of 24 h to achieve maximal RNA interference effect.

RT-PCR—RT-PCR was performed by using ProtoScript M-MuLV TaqRT-PCR Kit (New England Biolabs) following the manufacturer's instructions. The primers for SWI5 were 5'-TCTCTAGGACTGAACCAAGAC-3' (forward) and 5'-CTC-GCTGGAACTCTGTAGGTG-3' (reverse), yielding a 439-bp PCR product. The primers for MEI5 were 5'-CAAATTGGTG-AAGCAGGTTTCAAG-3' (forward) and 5'-CATTGGGATACCTTCCTCTGAG-3' (reverse), yielding a 623-bp PCR product.

Constructs—Human SWI5 (C9orf119) cDNA was obtained from OriGene Technologies (catalogue no. RG211457; RefSeq, NM_001040011). Human MEI5 (C10orf78) cDNA was obtained from human ORFeome collection (hORFeome version 5.1, GenBankTM accession no. BC020892). All cDNAs were subcloned into pDONR201 (Invitrogen) as entry clones and were subsequently transferred to gateway compatible destination vectors for the expression of N-terminal-tagged fusion protein. SFB (triple-epitope of S-protein, FLAG, and streptavidin binding peptide), Myc, MBP, and GST-tagged proteins were used in this study as described in the text. All point or deletion mutants were generated using the QuikChange site-directed mutagenesis kit (Stratagene) and verified by sequencing.

Binding Assays—For co-immunoprecipitation assays, constructs encoding SFB-tagged and Myc-tagged proteins were transiently co-transfected into 293T cells. Cells were lysed in NETN buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40) on ice for 30 min, cleared by centrifugation, and incubated with S protein beads for 2 h at 4 °C. Beads were washed, boiled in 2× Laemmli buffer, and separated on SDS-PAGE. Membranes were blocked in 5% milk in Tris-buffered saline/Tween buffer and then probed with antibodies as indicated. For direct binding assays, bacterially purified

MBP-tagged and GST-tagged proteins were incubated together in NETN buffer containing glutathione-agarose beads for 2 h at 4 °C. Beads were washed, boiled in 2× Laemmli buffer, and separated on SDS-PAGE.

Immunostaining—Cells cultured on coverslips were treated with ionizing radiation (IR) and then allowed to recover. Cells were then washed with PBS, pre-extracted with solution containing 0.5% Triton X-100 for 3 min and fixed with 3% paraformaldehyde for 12 min. Coverslips were washed with PBS and then immunostained with primary antibodies in 5% goat serum for 30 min at room temperature. Coverslips were washed and incubated with secondary antibodies conjugated with rhodamine or FITC for 30 min. Cells were subsequently stained with DAPI for the visualization of nuclear DNA. The coverslips were mounted onto glass slides with anti-fade solution and visualized under a Nikon ECLIPSE E800 fluorescence microscope with a Nikon Plan Fluor 40× oil objective lens (numerical aperture, 1.30) at room temperature. Cells were photographed and analyzed using a SPOT camera (Diagnostic Instruments, Inc.) and Adobe Photoshop software.

Homologous Recombination Assay—A U2OS cell clone stably expressing HR reporter direct repeat GFP was described previously (28). This reporter consists of two differentially mutated GFP genes oriented as direct repeats. Expression of I-SceI endonuclease will generate a site-specific DSB between the mutated GFP genes, which when repaired by gene conversion, results in a functional GFP gene. Briefly, 2 days after transfection with indicated siRNAs, 1×10^6 U2OS direct repeat GFP cells were electroporated with 20 μg of pCBASce, an I-SceI expression vector described previously (29). Cells were harvested 2 days after electroporation and subjected to flow cytometry analysis to determine percentages of GFP-positive cells, resulted from HR repair induced by I-SceI-induced DSBs.

Cell Survival Assay—A total of 1×10^3 cells were seeded onto a 60-mm dish in triplicate. Twenty-four hours after seeding, cells were irradiated by using a JL Shepherd Mark I-68A 137Cs-irradiator at indicated doses and incubated for 14 days. Resulting colonies were fixed and stained with Coomassie Blue. Numbers of colonies were counted using a GelDoc with Quantity One software (Bio-Rad).

RESULTS

SWI5-MEI5 Is an Evolutionarily Conserved Protein Complex—To identify human homologues of yeast Swi5 and Mei5, we used BLAST (Basic Local Alignment Search Tool) to search for human proteins with Swi5 or Mei5 domains, similar to budding yeast Sae3 or Mei5. Two uncharacterized proteins, C9orf119 and C10orf78, are found to contain Swi5 and Mei5 domains, respectively (Fig. 1A). The C9orf119 gene is located at chromosome 9q34.11. This gene encodes a protein of 235 amino acids. The C10orf78 gene is located at chromosome 10q25.1, which encodes a protein of 232 amino acids. Although the Swi5 domain sits at the C terminus of C9orf119, the Mei5 domain occupies a major portion of C10orf78. Here, we designated C9orf119 as human SWI5 and C10orf78 as human MEI5.

We selected several Swi5 and Mei5 homologues from different organisms and performed multiple sequence alignment using the ClustalW2 program (see Fig. 1A and supplemental

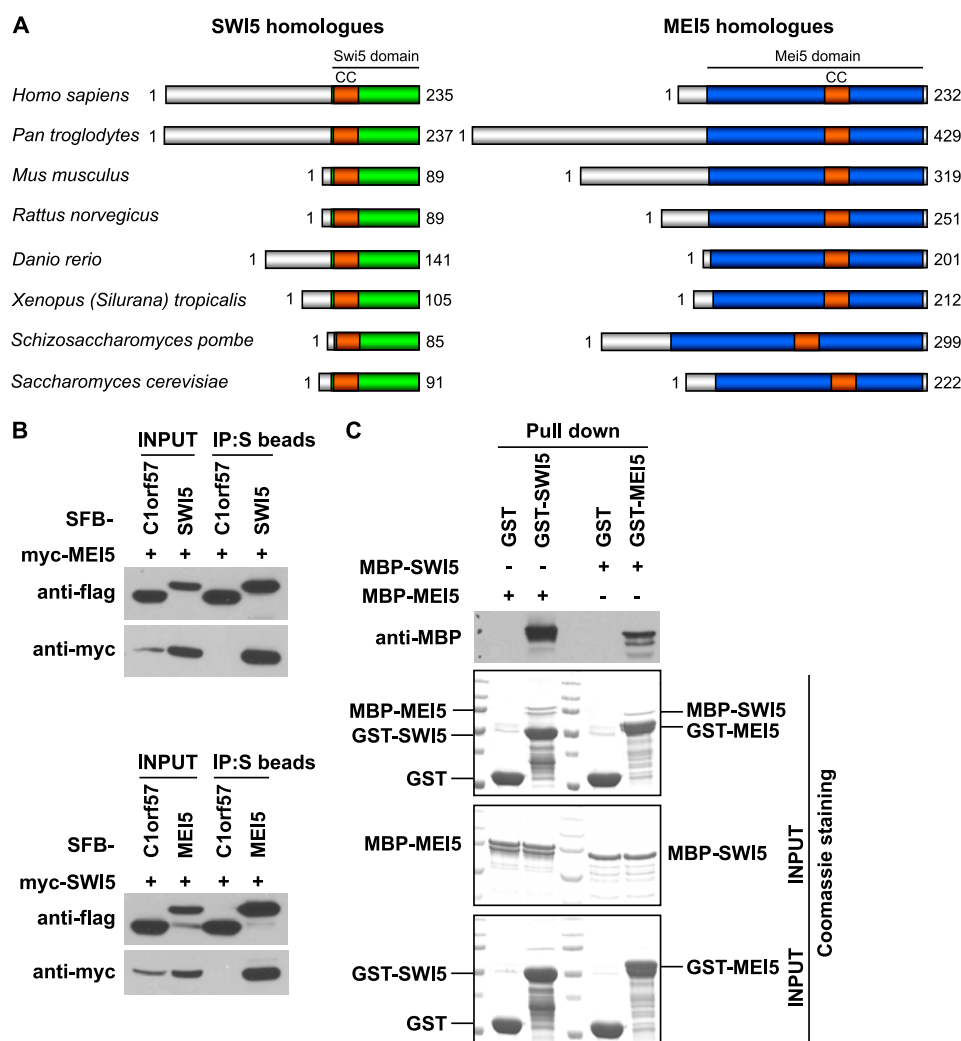


FIGURE 1. SWI5 and MEI5 form an evolutionarily conserved protein complex. A, all SWI5 or MEI5 orthologues contain a Swi5 or Mei5 domain. Conserved coiled-coil motifs (CC) are indicated within both Swi5 and Mei5 domains. The accession numbers of SWI5 orthologues are: NP_001035100.1 for *Homo sapiens*, XP_001144446.1 for *Pan troglodytes*, NP_780399.1 for *Mus musculus*, XP_575112.2 for *Rattus norvegicus*, XP_002663552.1 for *Danio rerio*, NP_001107337.1 for *Xenopus (Silurana) tropicalis*, NP_595453.1 for *Schizosaccharomyces pombe*, and NP_011947.2 for *Saccharomyces cerevisiae*. The accession numbers of MEI5 orthologues are: NP_660290.3 for *H. sapiens*, XP_001135759.1 for *P. troglodytes*, NP_080653.2 for *M. musculus*, NP_001041321.1 for *R. norvegicus*, NP_001076329.1 for *D. rerio*, XP_002936845.1 for *X. (Silurana) tropicalis*, NP_595668.1 for *S. pombe*, and NP_015204.1 for *S. cerevisiae*. B, the interaction between SWI5 and MEI5 was confirmed by co-immunoprecipitation (IP) experiments. 293T cells were transfected with plasmids encoding Myc-tagged SWI5 or MEI5 together with plasmids encoding SFB-tagged MEI5 or SWI5 as indicated. SFB-tagged C1orf57 was used here as a control. C, SWI5 and MEI5 directly interact with each other. MBP-tagged and GST-tagged proteins were expressed and purified from *E. coli*. Pull-down assays were performed by incubating purified proteins together with glutathione-agarose beads in NETN buffer. Beads were washed, associated proteins were eluted and detected by Coomassie staining and immunoblotting using antibodies as indicated.

Figs. 1 and 2). A conserved coiled-coil motif was identified in both Swi5 and Mei5 from different species (Fig. 1A and [supplemental Figs. 1 and 2](#)). As we will show below in Fig. 2, the coiled-coil motif of MEI5 is required for its interaction with SWI5, but the role of coiled-coil motif in SWI5 remains to be determined.

The SWI5-MEI5 counterpart in budding yeast, Sae3-Mei5, is only expressed during meiosis (20, 21, 30). When we searched human expressed sequence tag (EST) database using UniGene entry Hs.259594 for SWI5 and Hs.93667 for MEI5, we found that both SWI5 and MEI5 are expressed in testis and ovary, but they are also expressed in many other tissues (data not shown). Indeed, the expression of SWI5 and MEI5 was easily detected by RT-PCR in all the nonmeiotic cell lines tested so far (data not shown), suggesting that the expression of human SWI5 and MEI5 is not restricted to meiosis.

The SWI5 and MEI5 homologues in yeasts have been shown to form a stable complex *in vivo* and *in vitro*. To check whether human SWI5 and MEI5 also interact with each other, we first performed co-immunoprecipitation experiments using epitope-tagged SWI5 and MEI5. We found that SFB-tagged SWI5 strongly interacted with Myc-tagged MEI5, and the reverse experiment confirmed this result (Fig. 1B). C1orf57 is another uncharacterized protein in the database, and we used it here as an unbiased control. It did not bind to either SWI5 or MEI5 (Fig. 1B). Interestingly, we found that the expression of tagged SWI5 or MEI5 was greatly enhanced when they were co-expressed in the cell (Fig. 1B), which indicates that SWI5 and MEI5 form a stable complex *in vivo* and are mutually interdependent for their stability. To verify a direct interaction between SWI5 and MEI5, we expressed and purified MBP-tagged and GST-tagged SWI5 or MEI5 from *E. coli*. Pull-down

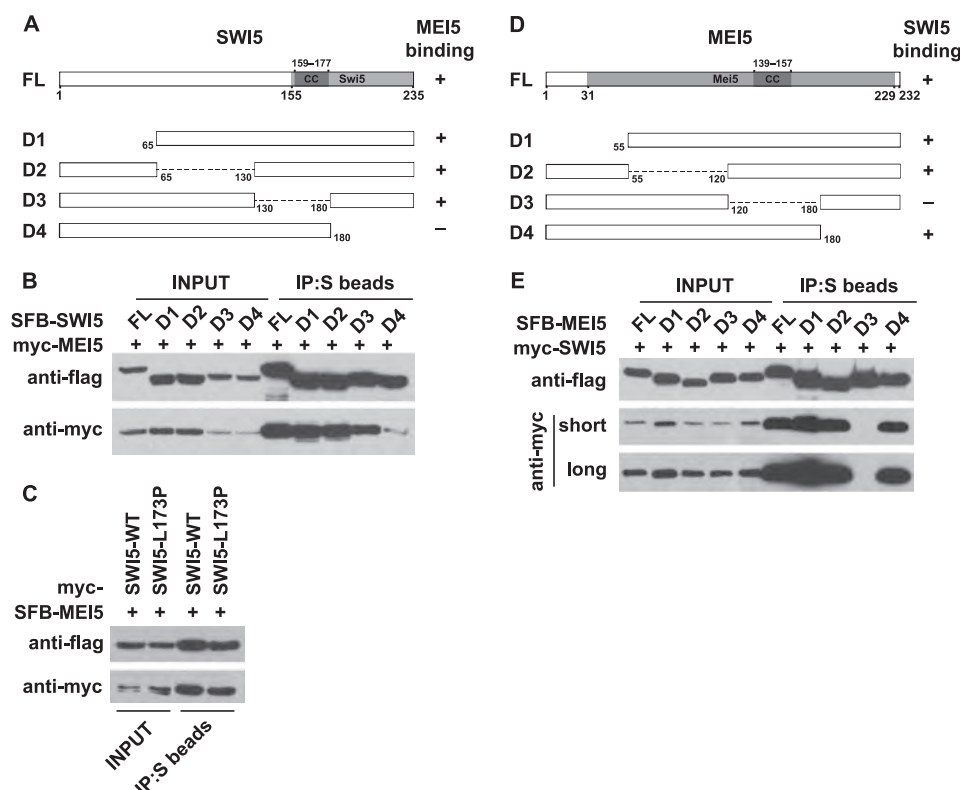


FIGURE 2. The C-terminal Swi5 domain of SWI5 and the middle coiled-coil region of MEI5 mediate the formation of this protein complex. A, shown is a schematic representation of full-length SWI5 and the mutants used in this study. Their abilities to bind to MEI5 are indicated. FL, full-length. B, the binding of SWI5 to MEI5 is greatly impaired with the deletion of the C-terminal Swi5 domain. C, a point mutation (L173P) within the coiled-coil motif of SWI5 does not affect its binding to MEI5. D, shown is a schematic representation of full-length MEI5 and the mutants used in this study. Their SWI5-binding abilities are indicated. E, a fragment containing coiled-coil motif of MEI5 is required for the binding of MEI5 to SWI5. 293T cells were transfected with plasmids encoding Myc-tagged and SFB-tagged proteins. Co-immunoprecipitation (IP) was performed as described under "Experimental Procedures."

assay demonstrated that SWI5 and MEI5 bind directly to each other (Fig. 1C). Together, these data support that human SWI5 and MEI5 form a stable complex.

C-terminal Swi5 Domain of SWI5 and Middle Coiled-coil Region of MEI5 Dictate Conserved Association of This Complex—To further determine the interaction between SWI5 and MEI5, we generated a series of truncation or internal deletion mutants of SWI5 and MEI5 (Fig. 2, A and D). As mentioned above, there are conserved coiled-coil motifs found in both Swi5 and Mei5 domains (supplemental Figs. 1 and 2). The coiled-coil motif is often involved in dimerization, oligomerization, and protein-protein interaction. However, we did not detect any homo-dimer or homo-oligomer formation for SWI5 or MEI5 (data not shown). The coiled-coil motif in SWI5 is not required for the binding of SWI5 to MEI5 because the SWI5 mutant deleted of this coiled-coil motif still associated with MEI5 (Fig. 2B), and a point mutation of a conserved residue in this coiled-coil domain of SWI5 (L173P) also failed to disrupt the binding of SWI5 to MEI5 (Fig. 2C). The MEI5-binding domain is located at the C terminus of SWI5, which constitutes the most conservative part of Swi5 domain (Fig. 2, A and B, and supplemental Fig. 1).

The binding assays performed between SWI5 and various mutants of MEI5 indicate that a fragment of MEI5 (residues 120–180), which contains the coiled-coil motif of MEI5, is required for the binding of MEI5 to SWI5 (Fig. 2, D and E), suggesting that the coiled-coil motif of MEI5 may be involved in the formation of this conserved SWI5-MEI5 complex in humans.

SWI5-MEI5 Is Important for Homologous Recombination Repair in Human Cells—Studies in budding and fission yeasts have revealed that the Swi5 and Mei5 are necessary for HR-mediated DNA repair and meiotic recombination, probably through interacting with the recombinases Rad51 and Dmc1 (20, 22). The broad expression profile of human SWI5 and MEI5 in various tissues and cell lines suggests a potential role of human SWI5-MEI5 in HR mediated DNA repair in mitotic cells. We first checked the interaction between SWI5-MEI5 and human RAD51. GST-tagged SWI5 or MEI5 and MBP-tagged RAD51 were expressed in *E. coli*, purified, and used in GST pull-down assays. We repeatedly detected the binding of SWI5 or MEI5 with RAD51, with the interaction between SWI5 and RAD51 much stronger than that of MEI5 and RAD51 (Fig. 3A).

The conserved SWI5-MEI5 complex formation and the direct binding of this complex to RAD51 suggest that the human SWI5-MEI5 complex may play a role in HR repair. To test whether SWI5 and MEI5 are required in HR mediated DNA repair, sets of siRNAs specifically targeting SWI5 or MEI5 were synthesized and introduced into U2OS cells. All four SWI5-specific siRNAs worked well because they led to significant down-regulation of SWI5 transcripts as detected by RT-PCR. The down-regulation of MEI5 by its siRNAs was also noticeable, although it was not as efficient as that of SWI5 siRNAs (Fig. 3B).

It is believed that at least a fraction of HR repair is initiated via DSB end resection, which generates ssDNA overhangs rapidly bound by RPA. Subsequently, the central recombinase enzyme

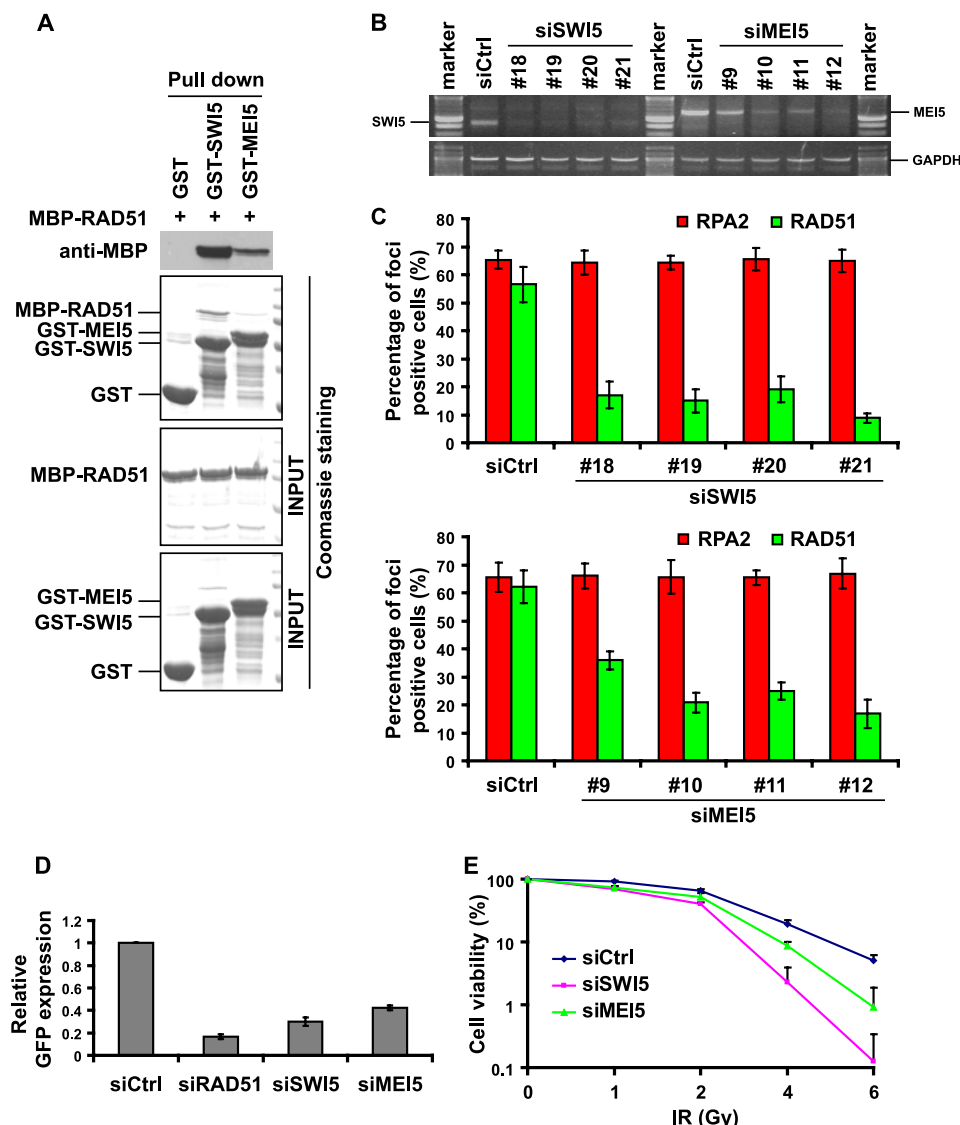


FIGURE 3. SWI5-MEI5 directly interacts with RAD51 and is involved in homologous recombination repair. *A*, SWI5 and MEI5, especially SWI5, can directly bind to RAD51. *B*, the efficiency of down-regulating the expression of their target genes by various siRNAs was evaluated by RT-PCR. *C*, down-regulation of SWI5 or MEI5 greatly impairs IR-induced RAD51 foci formation without influencing RPA foci formation. Immunostaining was performed 6 h after IR treatment with the indicated antibodies. *D*, decreasing of SWI5 or MEI5 impairs HR repair. U2OS direct repeat GFP cells were electroporated with pCBASce plasmids (see “Experimental Procedures” for details). The percentage of GFP-positive cells was determined by flow cytometry 48 h after electroporation. The data were normalized to those obtained from cells transfected with control siRNA (set as 1.0). Means and S.E. (error bars) shown are obtained from three independent experiments. *E*, cells with SWI5 or MEI5 down-regulation display increased IR sensitivity. Cell survival assays were performed as described under “Experimental Procedures.” Data were presented as means and S.D. (error bars) from three different experiments. Ctrl, control; Gy, gray.

RAD51, with the help of its accessory factors, displaces RPA from ssDNA to form a RAD51 filament, which starts homology search and HR repair. Thus, RPA and RAD51 foci formation can be used as readouts for two different steps during HR repair. Although RPA foci formation indicates the generation of ssDNA regions after DSB induction, the formation of RAD51 foci can be used as an indicator of actively ongoing HR repair process (26, 31). We checked both RPA and RAD51 foci formation after IR treatment in the control, SWI5- or MEI5-depleted U2OS cells. We did not detect any obvious changes in RPA foci formation following SWI5 or MEI5 down-regulation; however, RAD51 foci formation was greatly impaired in cells transfected with SWI5 or MEI5 siRNAs (Fig. 3C, also see supplemental Fig. 3 for representative immunostaining images).

Moreover, we examined HR efficiency using the established direct repeat GFP reporter system (28). In agreement with the results of reduced RAD51 foci formation, the efficiency of HR repair was clearly decreased in cells with SWI5 or MEI5 down-regulation (Fig. 3D). Consistently, down-regulation of SWI5 or MEI5 by siRNAs also resulted in increased cellular sensitivity to IR (Fig. 3E). Taken together, these data support an important role of the human SWI5-MEI5 complex in HR-mediated DNA repair.

DISCUSSION

In this study, we report the identification and characterization of the human SWI5-MEI5 complex. This complex directly binds to RAD51 and promotes RAD51 focus formation follow-

ing DNA damage. Together, these data support that SWI5-MEI5 is another mediator involved in homologous recombination repair in humans.

During the preparation of our article, the mouse homologues of SWI5-MEI5 (or Swi5-Sfr1) was identified (32). In that study, mouse Swi5-Sfr1 was identified as a complex required for genomic integrity with a specific role in the repair of DNA strand breaks (32). However, HR defects in Swi5^{-/-} and Sfr1^{-/-} embryonic stem cells were relatively mild (32). The variation in the severity of HR defect between human and mouse when SWI5-MEI5 is depleted could be due to different cells used in these studies. Alternatively, it may reflect the difference in the utilization of the SWI5-MEI5 complex in HR repair in embryonic stem cells *versus* adult somatic cells. Future experiments are needed to distinguish these possibilities.

The possible function of human SWI5-MEI5 in meiosis also needs to be investigated. It remains to be determined whether the human SWI5-MEI5 complex acts in meiosis and, if it does, whether it acts with DMC1, RAD51, or both. Considering that SWI5-MEI5 is the only human counterpart of the yeast Sae3-Mei5 complex, we speculate that SWI5-MEI5 may play important roles both in mitotic and meiotic homologous recombination reactions. Further experimentation on SWI5-MEI5 will reveal molecular mechanisms underlying HR process in humans, which is critical for the prevention of many human diseases including infertility and cancer.

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MDC1 collaborates with TopBP1 in DNA replication checkpoint control

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Human TopBP1 is a major player in the control of the DNA replication checkpoint. In this study, we identified MDC1, a key checkpoint protein involved in the cellular response to DNA double-strand breaks, as a TopBP1-associated protein. The specific TopBP1–MDC1 interaction is mediated by the fifth BRCT domain of TopBP1 and the Ser-Asp-Thr (SDT) repeats of

MDC1. In addition, we demonstrated that TopBP1 accumulation at stalled replication forks is promoted by the H2AX/MDC1 signaling cascade. Moreover, MDC1 is important for ATR-dependent Chk1 activation in response to replication stress. Collectively, our data suggest that MDC1 facilitates several important steps in both cellular DNA damage response and the DNA replication checkpoint.

Introduction

In eukaryotic cells, the DNA damage response helps to maintain genomic integrity. DNA damage induces signaling pathways that activate DNA repair processes and cell cycle checkpoints. The phosphoinositide kinase-related kinases ataxia telangiectasia mutated (ATM) and ATM and Rad3-related (ATR) are involved in DNA damage response and replication checkpoint control, respectively. ATM is activated primarily by DNA double-strand breaks (DSBs), whereas ATR responds principally to replication blockage or replication stress. In response to DNA DSBs, the histone variant H2AX is phosphorylated by ATM, which recruits a downstream checkpoint protein, mediator of DNA damage checkpoint protein 1 (MDC1), to sites of DNA damage. In addition, MDC1 is also phosphorylated on DNA damage and further facilitates the loading of the E3 ubiquitin ligase RNF8 to DSB sites. RNF8 ubiquitinates H2AX, and probably other substrates, and facilitates the accumulation of many DNA damage repair proteins at sites of DSBs (Wood and Chen, 2008; Yan and Jetten, 2008; Messick and Greenberg, 2009). The accumulation of these DNA damage repair proteins at DSB sites via the H2AX/MDC1-dependent pathway is generally believed to facilitate DNA damage repair and checkpoint control in response to DSBs.

A similar signal transduction pathway exists for cellular response to replication stress. We showed recently that both the replication checkpoint protein TopBP1 and a DNA helicase, BACH1 (also known as FANCF), are recruited to stalled replication forks, facilitating the accumulation of additional replication protein A (RPA)-coated single-stranded DNA (ssDNA) at stalled replication forks (Gong et al., 2010). This efficient accumulation of RPA-coated ssDNA leads to the assembly of multiprotein complexes, including ATR–ATR interacting protein (ATR–ATRIP), TopBP1, and Rad9–Hus1–Rad1 (dubbed as 9-1-1) at stalled replication forks, which is required for the activation of ATR kinase activity and for subsequent Chk1 phosphorylation and activation (Kumagai and Dunphy, 2006; Burrows and Elledge, 2008; Cimprich and Cortez, 2008; Yan and Michael, 2009).

Human TopBP1 and its orthologues in other organisms play important roles in DNA replication and replication checkpoint control (Saka et al., 1994; Wang and Elledge, 1999; Yamamoto et al., 2000; Mäkinen et al., 2001; Van Hatten et al., 2002; Yamane et al., 2002; Kim et al., 2005). It has been suggested that TopBP1 has acquired diverse functions by its abilities to interact with many binding partners via its multiple protein–protein interaction domains, including eight BRCA1 C-terminal (BRCT) phospho-peptide recognition motifs. For instance, TopBP1 regulates DNA replication initiation. Early studies in yeast suggested that this function of Dpb11, the yeast orthologue of TopBP1, can

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Abbreviations used in this paper: ATM, ataxia telangiectasia mutated; ATR, ATM and Rad3-related; co-IP, coimmunoprecipitation; DSB, double-strand break; HU, hydroxyurea; IP, immunoprecipitation; IR, ionizing radiation; MEF, mouse embryonic fibroblast; PCC, premature chromosome condensation; RPA, replication protein A; SFB, streptavidin-binding peptide; ssDNA, single-stranded DNA; WT, wild type.

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Supplemental Material can be found at:
<http://jcb.rupress.org/content/suppl/2011/04/11/jcb.201010026.DC1.html>

interact with Sld3 through BRCT1-2 of Dpb11 and with Sld2 through BRCT3-4 of Dpb11 (Tanaka et al., 2007; Zegerman and Diffley, 2007). More recently, Treslin/Ticrr has been shown to collaborate with TopBP1 in promoting replication initiation (Kumagai et al., 2010; Sansam et al., 2010). Although Treslin/Ticrr does not share any obvious sequence homology with yeast Sld2 or Sld3, the same N-terminal tandem BRCT1-2 domains are involved in this interaction, which suggests that the functions of TopBP1 are evolutionarily conserved.

TopBP1 also plays a key role in replication checkpoint control. An ATR-activating domain within TopBP1 interacts directly with ATR-ATRIP and thus activates ATR kinase activity (Kumagai et al., 2006). In addition, TopBP1 also interacts with the phosphorylated Rad9 tail of the 9-1-1 complex through its N-terminal tandem BRCT1-2 domains (Delacroix et al., 2007; Lee et al., 2007); this interaction is also required for Chk1 activation. The same N-terminal BRCT domains of TopBP1 interact with Rad9, NBS1, and (as recently shown) Treslin/Ticrr (Delacroix et al., 2007; Lee et al., 2007; Yoo et al., 2009), which indicates that the diverse roles of TopBP1 in replication and replication checkpoint control may be mediated by its distinct binding partners. Recently, we reported that TopBP1 associates with BACH1 through the very C-terminal tandem BRCT domains of TopBP1, which are required for early replication checkpoint control (Gong et al., 2010). However, we showed that BACH1 is not required for the accumulation of TopBP1 at stalled replication forks (Gong et al., 2010). Thus, despite all of these advances, we still do not know how TopBP1 accumulates at stalled replication forks. Although we showed that the fifth BRCT domain (BRCT5) of TopBP1 is required for TopBP1 focus formation after DNA damage (Yamane et al., 2002), the identity of an upstream regulator that would bind to TopBP1 BRCT5 and facilitate the recruitment of TopBP1 to DNA damage sites remains elusive.

In this study, we report a functional interaction between TopBP1 and MDC1. MDC1 is a large adaptor protein, best known for its roles in DNA damage response after DNA DSBs (Jungmichel and Stucki, 2010). MDC1 binds to the phosphorylated Ser139 site of H2AX (γ -H2AX) through its tandem BRCT domains, which further amplify DNA damage signals. MDC1 also binds to RNF8 and initiates an ubiquitination-mediated signaling cascade at DSB sites. Recently, we and others have shown that phosphorylation of the conserved Ser-Asp-Thr (SDT) repeats at the N terminus of MDC1 facilitates the recruitment and retention of NBS1 at DNA damage sites, thereby increasing the local concentration of the MRE11-RAD50-NBS1 (MRN) complex, which is required for intra-S phase checkpoint control after DNA DSBs (Chapman and Jackson, 2008; Melander et al., 2008; Spycher et al., 2008; Wu et al., 2008). Here, we describe a physical interaction between MDC1 and TopBP1 and suggest that MDC1 plays a similar, but unexpected, role in replication checkpoint control.

Results and discussion

TopBP1 accumulation at stalled replication forks requires TopBP1 BRCT5 domain

Previous work by our group documented that TopBP1 BRCT5 domain is important for TopBP1 focus formation in response

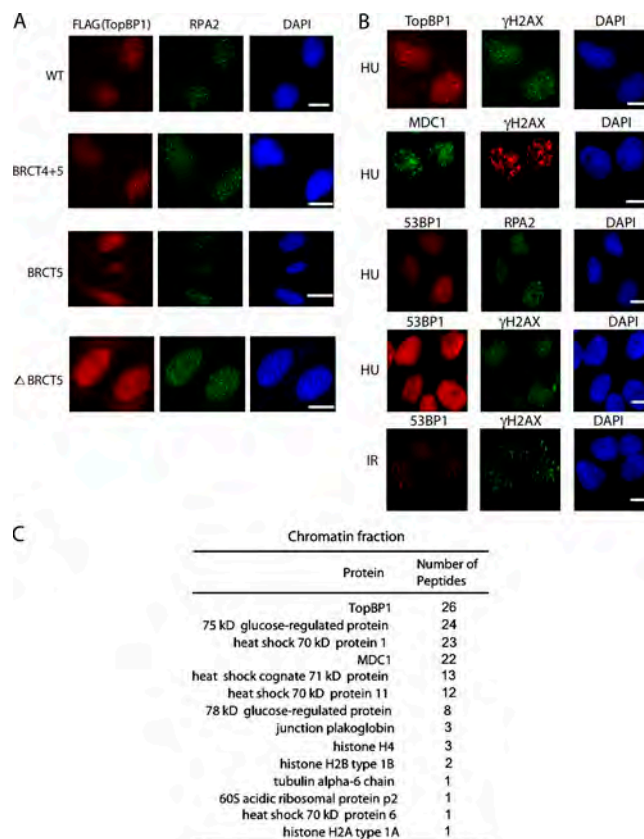


Figure 1. TopBP1 accumulation at stalled replication forks requires TopBP1 BRCT5 domain. (A) U2OS cells were transfected with plasmids encoding SFB-tagged WT or deletion mutants of TopBP1. Immunostaining was performed with the indicated antibodies in cells treated with 2 mM HU. (B) U2OS cells were treated with HU (top) or IR (bottom). After 2 h, cells were fixed and immunostaining was performed with the indicated antibodies. Bars, 10 μ m. (C) TopBP1 BRCT4+5-associated proteins in the chromatin fraction identified by mass spectrometric analysis.

to DNA damage (Yamane et al., 2002). To finely map the focus localization region of TopBP1, we generated several TopBP1 constructs. Similar to our previous results (Yamane et al., 2002), we found that deletion of TopBP1 BRCT5 domain abolished TopBP1 focus formation after hydroxyurea (HU) treatment, whereas normal focus localization was observed when a construct containing both the BRCT4 and BRCT5 domains (BRCT4+5) of TopBP1 was used (Fig. 1 A). We found that a region containing BRCT5 domain of TopBP1 (residues 545–722) is sufficient for TopBP1 focus formation after HU treatment (Fig. 1 A). HU treatment should lead to replication stress only in S phase cells. Indeed, we found that HU-induced TopBP1 focus formation was restricted to S phase cells, which were also positive for cyclin A staining (Fig. S1 A). To distinguish whether HU-induced TopBP1 focus formation represents stalled replication forks or fork-derived DNA DSBs, we used 53BP1 as a marker of DNA DSBs and found that under our experimental condition (after 2 h of treatment with 2 mM HU), HU treatment did not induce a significant amount of DNA DSBs (Fig. 1 B). This result was further confirmed by a time-course experiment after HU treatment (Fig. S1 B).

Next, we wanted to identify the upstream signaling molecules that facilitate TopBP1 accumulation at stalled replication forks.

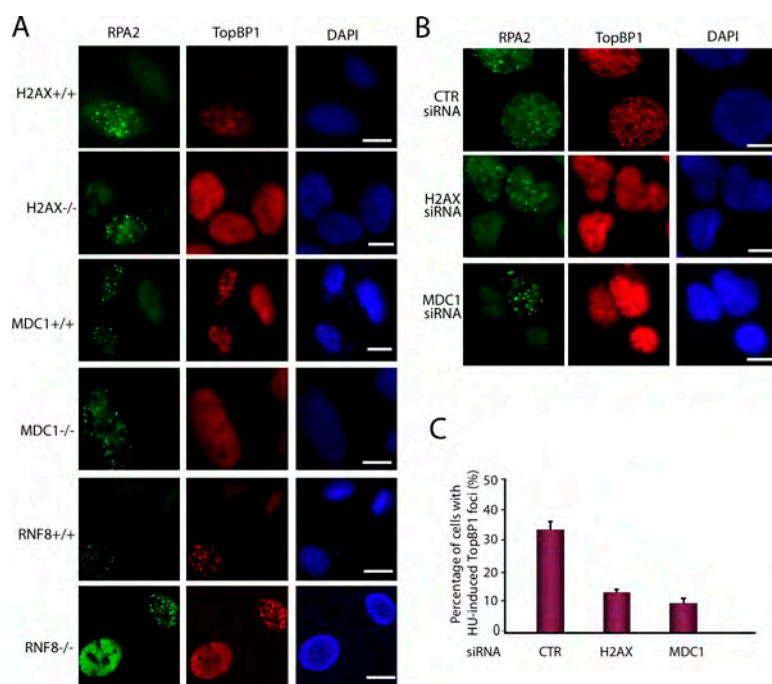


Figure 2. TopBP1 focus formation depends on H2AX/MDC1 but not on RNF8 after replication stress. (A) Cells deficient in H2AX, MDC1, and RNF8, and their WT counterparts were treated with HU, and immunostaining experiments were performed with anti-TopBP1 and anti-RPA2 antibodies. (B) U2OS cells were transfected with control siRNA (CTR), H2AX-specific, or MDC1-specific siRNA. Cells were treated with HU, fixed, and immunostained with anti-TopBP1 and anti-RPA2 antibodies. Bars, 10 μ m. (C) Percentages of cells stained positive for TopBP1 foci were determined in cells transfected with the indicated siRNAs. Data are presented as mean \pm SD (error bars) from three different experiments.

Thus, we performed tandem affinity purification using lysates derived from 293T cells stably expressing triple-tagged (S protein, FLAG, and streptavidin-binding peptide [SFB]-tagged) BRCT4+5 domain of TopBP1. Surprisingly, mass spectrometric analysis showed that MDC1 was the major TopBP1-associated protein in the chromatin fraction (Fig. 1 C), which suggests that MDC1 may be involved in TopBP1 accumulation at stalled replication forks.

TopBP1 focus localization after replication stress requires a H2AX/MDC1-dependent signaling pathway

We next explored whether MDC1 might be essential for the focus accumulation of TopBP1 at stalled replication forks after replication stress. We used a panel of mouse embryonic fibroblast (MEF) cell lines deficient in MDC1 or MDC1-associated molecules. We found that TopBP1 focus formation was greatly reduced in H2AX^{-/-} and MDC1^{-/-} MEFs compared with their wild-type (WT) counterparts (Fig. 2 A), which indicates that HU-induced focus formation of TopBP1 requires both H2AX and MDC1. In contrast, normal TopBP1 focus formation was observed in both RNF8^{-/-} MEFs and their WT counterparts (Fig. 2 A), which suggests that the RNF8-dependent ubiquitination cascade is not involved in TopBP1 accumulation after replication stress.

Similarly, we observed that HU-induced TopBP1 focus formation was reduced in U2OS cells with H2AX or MDC1 knock-down (Fig. 2, B and C; and Fig. S2 C). Collectively, these data demonstrate that TopBP1 acts downstream of H2AX and MDC1, but is independent of RNF8, in response to replication stress.

MDC1 interacts with TopBP1

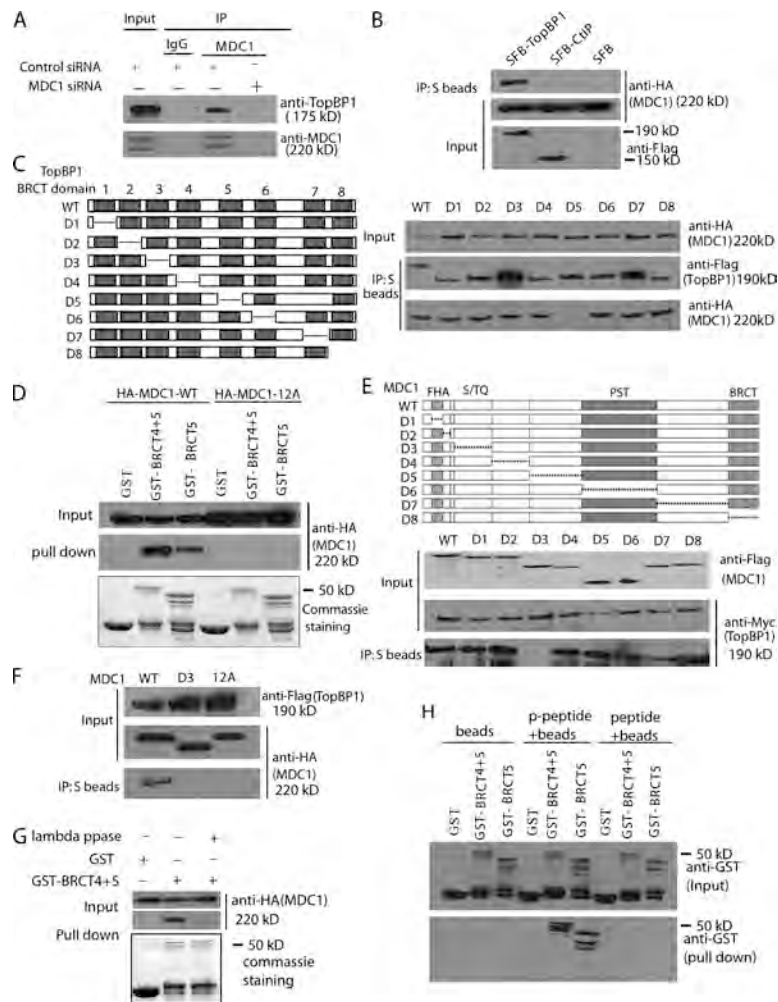
In addition to identifying MDC1 as a major TopBP1-associated protein, we also repeatedly identified TopBP1 as a major MDC1-associated protein in the chromatin fraction by tandem affinity

purification using lysates derived from 293T cell lines stably expressing SFB-tagged human MDC1 (unpublished data). Collectively, these results provide support for a physical interaction between TopBP1 and MDC1. We performed coimmunoprecipitation (co-IP) experiments and confirmed an interaction not only between endogenous proteins (Fig. 3 A), but also between over-expressed MDC1 and TopBP1 (Fig. 3 B), which suggests that TopBP1 is a bona fide MDC1-interacting protein. Moreover, knocking down the other MDC1 binding partner, NBS1, did not affect the binding of MDC1 with TopBP1 (Fig. S3 A).

To determine the regions on TopBP1 required for its interaction with MDC1, we subjected SFB-tagged WT TopBP1 and a series of TopBP1 internal-deletion mutants (Fig. 3 C, left) to co-IP experiments with full-length HA-tagged MDC1. Only deletion of TopBP1 BRCT5 domain led to a dramatic decrease in the TopBP1–MDC1 interaction (Fig. 3 C, right), confirming that TopBP1 BRCT5 domain is responsible for the binding of TopBP1 to MDC1. Furthermore, using bacterially expressed and purified proteins, we found that both TopBP1 BRCT4+5 and TopBP1 BRCT5 domains bound to MDC1 (Fig. 3 D). To determine which residues in the BRCT5 domain of TopBP1 are required for the association of TopBP1 with MDC1, we mutated two highly conserved Trp residues in BRCT5 (W711R and W720R). These mutants disrupted the TopBP1–MDC1 interaction and accordingly abolished TopBP1 focus formation (Fig. S3, B and C).

Next, we sought to define the TopBP1 binding regions on MDC1. Again, a series of MDC1 internal-deletion mutants were coexpressed with Myc-tagged TopBP1 in 293T cells. The interaction between MDC1 and TopBP1 was dramatically decreased by D3, a mutant with deletion of a region of MDC1 that is enriched for SDT repeats (Fig. 3 E). This result indicates that the SDT repeats of MDC1 may be involved in its interaction with TopBP1, just as they are involved in its interaction with NBS1 (Chapman and Jackson, 2008; Melander et al., 2008; Spycher et al., 2008;

Figure 3. TopBP1 interacts with MDC1. (A) Endogenous interaction between TopBP1 and MDC1. HeLa cells were transfected with control siRNA or MDC1-specific siRNA. Control or anti-MDC1 immunoprecipitates were immunoblotted with the indicated antibodies. (B) TopBP1 binds specifically to MDC1. Constructs encoding SFB-tagged TopBP1, CtIP, or vector alone were cotransfected with plasmids encoding HA-tagged MDC1. Immunoprecipitation (IP) and immunoblotting were performed as indicated. (C) Schematic presentation of WT and deletion mutants of TopBP1 used in this study (left). 293T cells were transfected with plasmids encoding HA-tagged MDC1 together with plasmids encoding WT or deletion mutants of SFB-tagged TopBP1. IP reactions were conducted using S protein beads and then subjected to Western blotting using the indicated antibodies (right). (D) Beads coated with bacterially expressed GST, GST fusion of TopBP1 BRCT4+5 domains, or TopBP1 BRCT5 domain were incubated with cell lysates containing exogenously expressed HA-tagged WT MDC1. Immunoblotting experiments were performed using the indicated antibodies (top). (E) Schematic diagram of WT and deletion mutants of MDC1 used in this study (top). 293T cells were transfected with plasmids encoding Myc-tagged TopBP1 together with plasmids encoding WT or deletion mutants of SFB-tagged MDC1. IP reactions were conducted using S protein beads and then subjected to Western blot analyses using antibodies as indicated. (F) TopBP1 binds to the phosphorylated SDT repeats of MDC1. 293T cells were transfected with plasmids encoding HA-tagged WT, D3 mutant, or 12A mutant of MDC1 together with plasmids encoding SFB-tagged TopBP1. IP reactions were conducted using S protein beads and then subjected to Western blotting using the indicated antibodies. (G) Extracts prepared from 293T cells expressing HA-tagged MDC1 were mock-treated or treated with λ phosphatase. Extracts were then incubated with 10 μ g of bacterially expressed and purified GST or GST-BRCT4+5 fusion proteins immobilized on glutathione agarose beads for 2 h at 4°C. The complex was separated by SDS-PAGE, and the amount of MDC1 that bound specifically to TopBP1 BRCT5 domain was evaluated by immunoblotting. (H) Phosphorylated or control MDC1 peptides were incubated with purified GST, or GST-BRCT4+5 or GST-BRCT5 fusion proteins. Input (top) and GST fusion proteins associated with peptides (bottom) were assessed by immunoblotting using anti-GST antibodies.



Wu et al., 2008). These SDT repeats are phosphorylated by CK2 kinase (Chapman and Jackson, 2008; Melander et al., 2008; Spycher et al., 2008; Wu et al., 2008). If TopBP1 binds to these phosphorylated repeats of MDC1, we would expect that a 12A mutant of MDC1, in which the Ser/Thr residues in all six SDT repeats were changed to Ala, would abolish the MDC1–TopBP1 interaction. Indeed, we found this to be the case (Fig. 3, D and F). In addition, the binding between MDC1 and TopBP1 was dramatically decreased when extracts were pretreated with λ phosphatase (Fig. 3 G). Moreover, only biotinylated phosphopeptides containing the consensus (p)SD(p)TDXE motif of MDC1, but not unphosphorylated peptides with the identical sequence, could pull down TopBP1 BRCT4+5 or TopBP1 BRCT5 fusion proteins (Fig. 3 H). Together, these data indicate that TopBP1 associates only with MDC1 that is phosphorylated at these conserved SDTD motifs.

Both TopBP1 and MDC1 are required for ATR activation in response to replication stress

Given that TopBP1 focus formation requires MDC1, we next determined whether the TopBP1-binding region on MDC1 is also required for TopBP1 accumulation in response to replication

stress. We transfected HeLa cells with FLAG-tagged siRNA-resistant WT MDC1, D3 mutant, or 12A mutant of MDC1. After siRNA-mediated depletion of endogenous MDC1, HU-induced TopBP1 focus formation was observed only in the cells reconstituted with WT MDC1 and not in the cells reconstituted with D3 mutant or 12A mutant of MDC1 (Fig. 4 A), which indicates that these SDT repeats of MDC1 are required for the TopBP1 focus formation after replication stress.

TopBP1 is required for Chk1 activation after replication stress (Burrows and Elledge, 2008). Although MDC1 is clearly involved in DNA damage response, its function in the replication stress pathway remains to be determined. We first examined the role of MDC1 in Chk1 phosphorylation after replication stress. Consistent with previous results (Kim et al., 2005), we found that depletion of TopBP1 inhibited HU-induced Chk1 phosphorylation (Fig. 4 C). MDC1-depleted cells also exhibited obvious reductions in HU-induced Chk1 and RPA2 phosphorylation, whereas NBS1-depleted cells displayed normal Chk1 phosphorylation in response to HU (Fig. 4 B).

Although the expression of siRNA-resistant WT TopBP1 completely restored Chk1 activation in cells depleted of endogenous TopBP1, reconstitution with a TopBP1 mutant with deletion of BRCT5 domain failed to rescue HU-induced Chk1

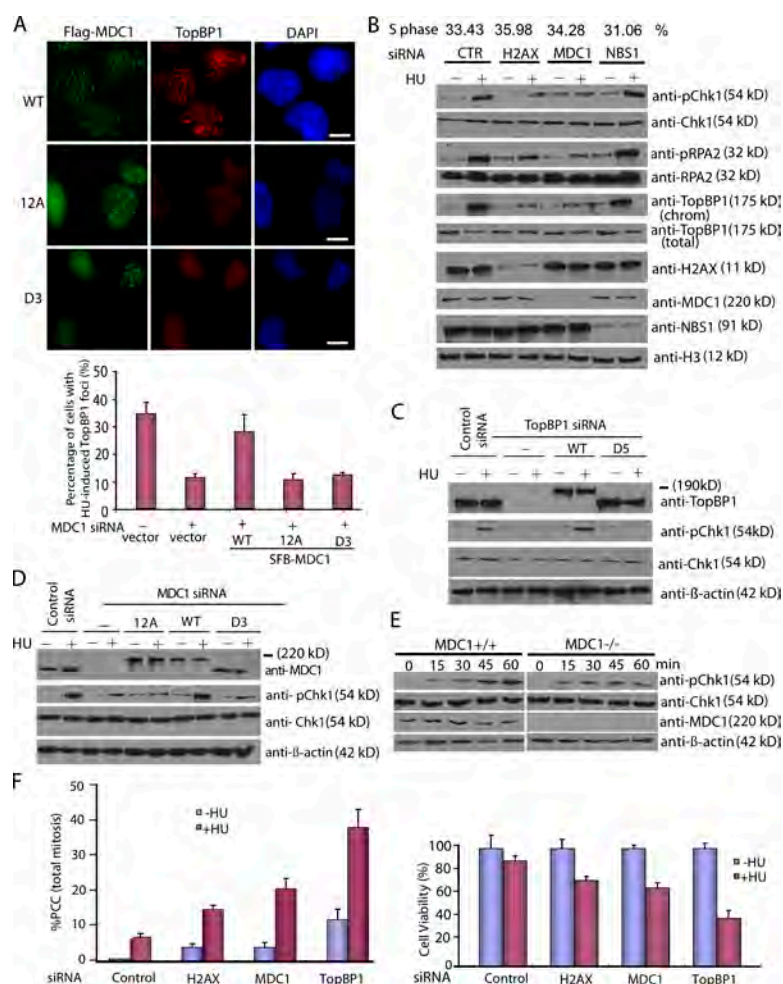


Figure 4. The TopBP1-MDC1 interaction is required for replication checkpoint control. (A) The SDT repeats of MDC1 are required for TopBP1 focus formation in response to HU. HeLa cells were transfected with FLAG-tagged siRNA-resistant WT, D3 mutant, or 12A mutant of MDC1, and with MDC1 siRNA twice with a 24-h interval. Cells were selected with puromycin for 48 h and then treated with HU. Immunostaining experiments were performed with anti-FLAG and anti-TopBP1 antibodies. Percentages of cells stained positive for TopBP1 foci were determined. Data are presented as mean \pm SD from three different experiments. Bars, 10 μ m. (B) Both H2AX and MDC1 are required for Chk1 activation in response to replication stress. U2OS cells transfected with control siRNA, H2AX siRNA, MDC1 siRNA, or NBS1 siRNA were mock-treated or treated with HU and harvested 1 h later. Cell lysates were prepared and immunoblotted with antibodies as indicated. (C) The interaction between TopBP1 and MDC1 is required for Chk1 activation. U2OS cells or U2OS cells stably expressing siRNA-resistant WT or D5 deletion mutant of TopBP1 were transfected with TopBP1 siRNA. 72 h after initial siRNA transfection, cells were treated with HU and collected 1 h later. Cell lysates were immunoblotted with the indicated antibodies. (D) HeLa cells were transfected with FLAG-tagged siRNA-resistant WT, D3 mutant, or 12A mutant of MDC1, and with MDC1 siRNA twice with a 24-h interval. Cells were selected with puromycin for 48 h and then treated with HU and collected 1 h later. Cell lysates were immunoblotted with the indicated antibodies. (E) MDC1^{-/-} MEFs and WT control MEFs treated with HU for different time periods and p-Chk1 levels were determined by Western blotting. (F, left) MDC1 prevents PCC after replication stress. HeLa cells were transfected with control siRNA, TopBP1 siRNA, or MDC1 siRNA and then treated with 2 mM HU and 200 ng/ml nocodazole for 20 h. Mitotic spreads were prepared, and percentages of cells containing PCC were evaluated under the microscope. Data are presented as mean \pm SD (error bars) from three independent experiments. (F, right) HeLa cells were arrested by HU for 12 h and released by changing with fresh medium. Cell survival after HU treatment was measured with clonogenic assay. Data are presented as mean \pm SD (error bars) from three independent experiments.

phosphorylation (Fig. 4 C). Furthermore, the expression of siRNA-resistant WT MDC1 fully rescued Chk1 activation in MDC1-depleted cells, whereas the expression of siRNA-resistant D3 mutant or 12A mutant of MDC1 failed to rescue the Chk1 phosphorylation defect after HU treatment (Fig. 4 D). These data indicate that the TopBP1-MDC1 interaction plays an important role in Chk1 activation after replication stress.

To further explore whether MDC1 is required for initial ATR activation or for signal amplification, we performed a detailed time-course experiment. MDC1^{-/-} MEF cells and WT control MEF cells were treated with HU for different time periods, and Chk1 phosphorylation levels were determined by Western blotting. Chk1 phosphorylation was increased after 15 min of HU treatment in both MDC1^{-/-} MEF cells and WT control MEF cells (Fig. 4 E). However, the Chk1 phosphorylation level kept on increasing continuously in WT cells but not in MDC1^{-/-} MEF cells. These results indicate that MDC1 is not required for initial ATR activation, but is involved in the amplification of ATR signaling after replication stress.

It is well established that replication checkpoint defects that abrogate the ATR-Chk1 pathway would lead to premature chromosome condensation (PCC; Nghiem et al., 2001). TopBP1- or MDC1-depleted HeLa cells displayed a substantial increase of PCC after HU treatment compared with cells transfected with control siRNA (Fig. 4 F, left). Moreover, TopBP1 or MDC1

depletion also reduced cell survival after HU treatment (Fig. 4 F, right). These results confirm that MDC1 is involved in replication checkpoint control.

Our data presented here are different from some of the observations recently described by another group (Cescutti et al., 2010). As we demonstrated in a previous study, recruitment of TopBP1 to sites of replication stress does not require the very C-terminal tandem BRCT domains (Gong et al., 2010). Moreover, as presented here, we took an unbiased approach and identified MDC1 as a TopBP1-associated protein. Our follow-up studies fully supported our initial finding and established that a physical interaction between TopBP1 and MDC1 is required for the stable accumulation of TopBP1 at sites of replication stress. We did not recover any 53BP1 peptides when we performed mass spectrometric analysis of TopBP1 BRCT5-associated proteins (Fig. 1 C), raising the possibility that the 53BP1-TopBP1 interaction may be relatively weak and thus insufficient to recruit TopBP1 to DNA damage sites. Indeed, we showed that ionizing radiation (IR) or HU-induced TopBP1 focus formation was easily detected in 53BP1^{-/-} MEFs (Fig. S3 D), which suggests that 53BP1 does not play a major role in recruiting TopBP1 after IR or HU treatment. A possible explanation for some of the conflicts between our data and those of Cescutti et al. (2010) is that the experiments were conducted in different ways (i.e., our experimental condition is after 2 h of HU treatment) and/or how the conclusions were deduced.

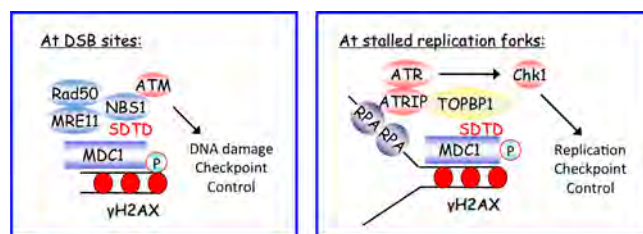


Figure 5. **A proposed model of replication checkpoint control.** This model involves a signal amplification step mediated by H2AX and MDC1 (see text for details).

MDC1 is best known for its role in cellular response to DNA DSBs. In this role, MDC1 binds to phosphorylated H2AX to amplify DNA damage signals. In addition, MDC1 interacts with NBS1 and is required for the retention of NBS1 at sites of DNA breaks (Chapman and Jackson, 2008; Melander et al., 2008; Spycher et al., 2008; Wu et al., 2008). The findings we report here agree with these results of earlier studies. In addition, this study has also clarified a role of H2AX in replication checkpoint control. Although we reported several years ago that H2AX is phosphorylated by ATR after replication stress (Ward and Chen, 2001), the exact role of H2AX phosphorylation in replication checkpoint control was not known before the current study. We propose that γ -H2AX plays an indirect role in the recruitment of TopBP1 via its direct binding to MDC1. We propose that, similar to their roles at DSB sites, γ -H2AX and MDC1 are also involved in the amplification of replication stress signals (our working model is presented in Fig. 5). In essence, ssDNA region coated by RPA at stalled replication forks is right next to the double-stranded DNA region coated by H2AX and MDC1. These two molecules are involved in the amplification of replication stress signals. At stalled replication forks, initial phosphorylation of H2AX by ATR or other related kinases triggers the recruitment of MDC1, which then leads to the accumulation of TopBP1 at stalled replication forks via a direct protein–protein interaction. The role of H2AX and MDC1 is to increase the local concentration of TopBP1 at and near stalled replication forks, and therefore facilitate the efficient activation of ATR kinase activity and, subsequently, Chk1 phosphorylation at stalled replication forks. Further analyses of these key molecules involved in DNA damage and replication checkpoint controls will provide insights into the interplay between these two major checkpoint pathways, which are critical for the maintenance of genomic integrity and for tumor suppression.

Materials and methods

Cell culture and plasmids

293T, U2OS, and HeLa cells were maintained in RPMI 1640 medium. MEFs cells were cultivated in DME medium. All media were supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin at 37°C in a humidified incubator with 5% CO₂ (vol/vol). H2AX^{-/-}, MDC1^{-/-}, RNF8^{-/-}, and their respective WT MEFs have been described previously (Lou et al., 2006; Huen et al., 2007). TopBP1 or MDC1 cDNA was cloned or subcloned using Gateway technology (Invitrogen). All internal-deletion mutants were generated by site-directed mutagenesis and verified by sequencing. We used a BRCT5 domain of TopBP1 (residues 545–722) that contained both BRCT5 domain and the region between BRCT4 domain and BRCT5 domain. The construct containing HA-tagged 12A mutant of

MDC1 was a gift from J. Lukas (Institute of Cancer Biology, Danish Cancer Society, Copenhagen, Denmark).

Antibodies

Rabbit polyclonal anti-TopBP1 and anti-MDC1 antibodies have been described previously (Yamane et al., 2002; Kim et al., 2005; Lou et al., 2006). Monoclonal anti-Flag M2, anti-HA, and anti- β -actin antibodies were obtained from Sigma-Aldrich. The anti-Myc (9E10) antibody was obtained from Covance. p-DNA-PK antibody was provided by D.J. Chen (University of Texas Southwestern Medical School, Dallas, TX).

Co-IP and Western blotting

Cells were lysed with NTEN buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, and 0.5% Nonidet P-40) containing protease inhibitors on ice for 20 min. The soluble fractions were collected after centrifugation and incubated for 3 h at 4°C with either protein A agarose beads coupled with anti-TopBP1, anti-MDC1 antibodies, or S protein agarose (EMD). The precipitates were then washed and boiled in 2× SDS loading buffer. Samples were resolved on SDS-PAGE and transferred to polyvinylidene fluoride membrane, and immunoblotting was performed with antibodies as indicated.

RNA interference

HeLa cells were transfected twice with a 24-h interval with the indicated siRNAs using Oligofectamine (Invitrogen) according to the manufacturer's instructions. siRNAs against human TopBP1 or MDC1 have been described previously (Yamane et al., 2002; Kim et al., 2005; Lou et al., 2006). The sequence of control siRNA was 5'-UUCAAUAAUUCUUGAGGUU-3'.

Tandem affinity purification

293T cells stably expressing SFB-TopBP1-BRCT4+5 or SFB-MDC1 were used for tandem affinity purification. Those stable cells were lysed with NTEN buffer (see Co-IP and Western Blotting) on ice for 20 min. After cell debris was removed by centrifugation, crude lysates were cleared by centrifugation. The pellets were suspended in nuclease buffer (10 mM Hepes, pH 7.4, 10 mM KCl, 0.5 mM MgCl₂, 2 mM CaCl₂, and 1 μ g/ml of each of pepstatin A and aprotinin) supplemented with 150 U/ml micrococcal nuclease S7 (Roche) and incubated in a 37°C water bath for 5 min until the suspension turned cloudy. Then the chromatin fraction was collected by centrifugation, and the supernatants were incubated with streptavidin Sepharose beads (GE Healthcare) for 3 h at 4°C. The bead-bound proteins were washed three times with NTEN buffer and eluted twice with 2 mg/ml biotin (Sigma-Aldrich) for 1 h at 4°C. The eluates were combined and then incubated with S protein agarose (EMD) for 3 h at 4°C. Beads were washed three times with NTEN buffer. The proteins bound to S protein agarose beads were separated by SDS-PAGE and stained with Coomassie blue. The eluted proteins were identified by mass spectrometric analysis (Taplin Biological Mass Spectrometry Facility, Harvard University).

Immunofluorescence staining

Cells grown on coverslips were mock-treated or treated with 2 mM HU for 2 h. Cells were fixed in 3% paraformaldehyde for 10 min and then permeabilized in 0.5% Triton X-100-containing solution for 5 min on ice. For immunostaining with TopBP1 antibody, cells were fixed in a mixture of acetone and methanol (1:1) at -20°C for 12 min. Cells were incubated with primary antibodies diluted in 5% goat serum at 37°C for 30 min. Cells were washed twice with PBS and then incubated with either fluorescein isothiocyanate (FITC)-conjugated or rhodamine-conjugated secondary antibodies at 37°C for 30 min. Nuclei were counterstained with DAPI. The coverslips were mounted onto glass slides with anti-fade solution and visualized at RT using a fluorescence microscope (Eclipse E800; Nikon) with a 60× NA 1.3 oil objective lens. Images were photographed and analyzed using a Spot 2 Megasample camera (Diagnostic Instruments, Inc.) and Photoshop software (Adobe).

Mitotic spreads

Evidence of premature mitosis in damaged cells relies primarily on the appearance of PCC. Mitotic spreads were prepared. In brief, HeLa cells were transfected with control siRNAs or siRNAs against human TopBP1 or MDC1. Then, 48 h after the first transfection, 2 mM HU and 200 ng/ml nocodazole were added. Cells were harvested for chromosome preparation using a standard protocol 6–8 h after treatment with colcemid treatment (50 ng/ml). Cells were incubated in 0.075 M KCl at 37°C for 20 min and then fixed by multiple changes of Carnoy's fixative (3:1 methanol/acetic acid). Cells were dropped onto slides and stained with Giemsa. PCC was scored as described previously (Nghiem et al., 2001).

GST pull-down assay

GST fusion proteins were expressed in *Escherichia coli* and purified as previously described (Hofer et al., 1994). GST fusion proteins were immobilized on glutathione-Sepharose 4B beads and incubated with lysates prepared from cells transiently transfected with plasmids encoding the indicated proteins. The samples were subjected to SDS-PAGE and analyzed by Western blotting.

Online supplemental material

Fig. S1 illustrates that HU-induced TopBP1 and MDC1 focus formation occurs at stalled replication forks but not at fork-derived DNA DSBs. Fig. S2 confirms that TopBP1 acts downstream of H2AX and MDC1 in response to replication stress. Fig. S3 shows the phosphorylation-specific interaction between MDC1 and TopBP1. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.201010026/DC1>.

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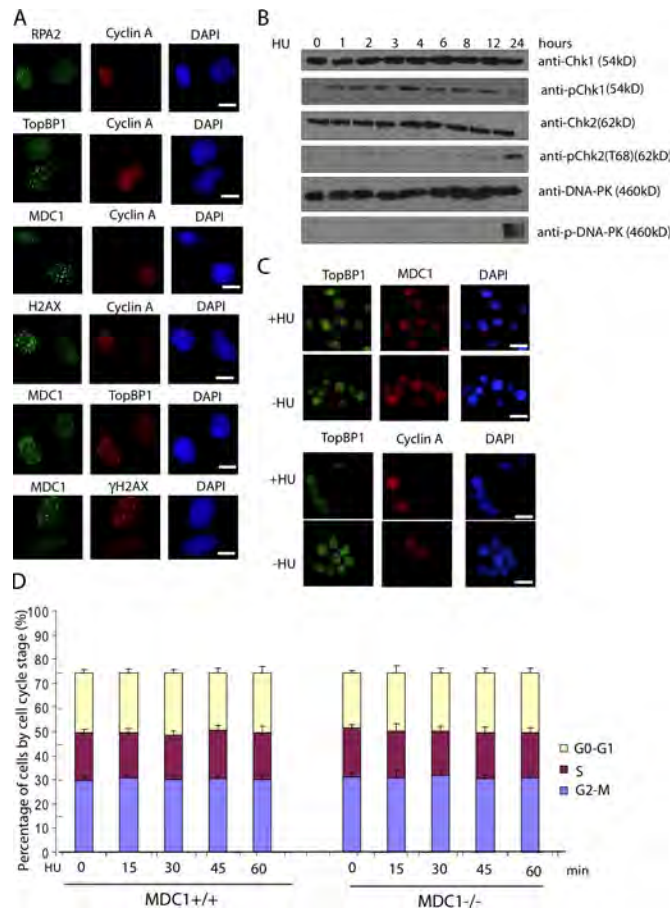


Figure S1. **HU-induced TopBP1 and MDC1 focus formation occurs at stalled replication forks.** (A) HeLa cells were treated with 2 mM HU for 2 h, and immunostaining was conducted using anti-cyclin A and other antibodies as indicated. Bars, 10 μ m. (B) HeLa cells were mock-treated or treated with HU for the indicated time periods (1–24 h), and cell lysates were subjected to Western blotting using antibodies as indicated. (C) HeLa cells were treated with 2 mM HU for 2 h, and immunostaining was performed using the indicated antibodies. Bars, 10 μ m. (D) MDC1^{-/-} and WT control MEFs were treated with HU as shown in Fig. 4 E. Samples were taken at the indicated time points and analyzed by FACS. Data are presented as mean \pm SD (error bars) from three different experiments.

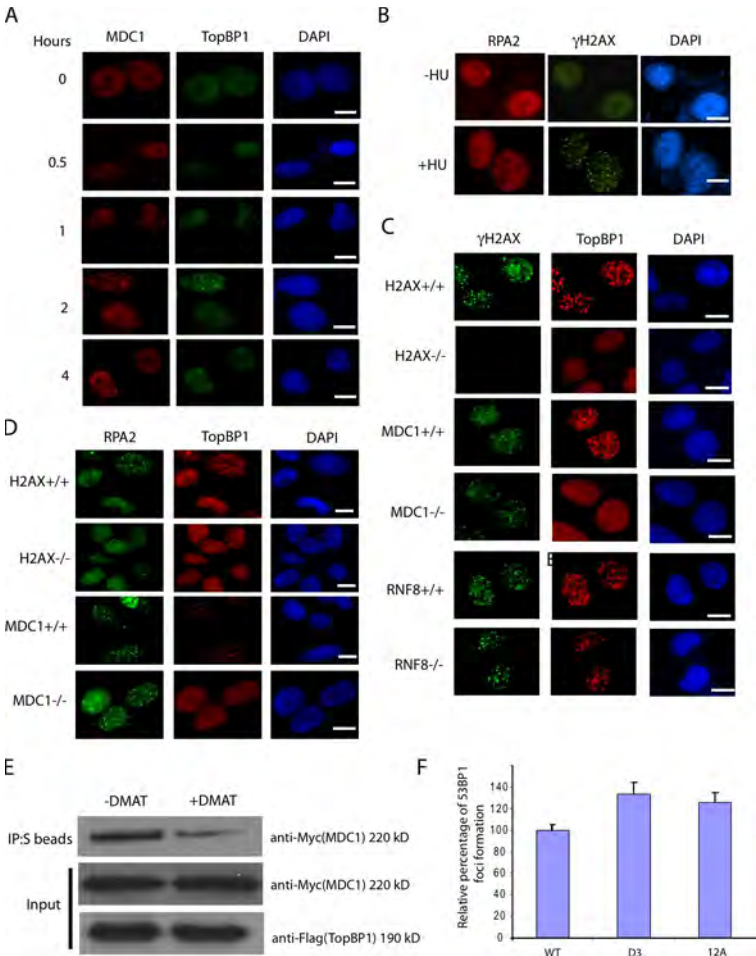


Figure S2. TopBP1 acts downstream of H2AX and MDC1 in response to replication stress. (A) U2OS cells were treated without or with 2 mM HU at different time points. Immunostaining experiments were performed with the indicated antibodies. (B) U2OS cells were treated without or with 2 mM HU for 2 h. Immunostaining experiments were performed with the indicated antibodies. (C) Cells deficient in H2AX, MDC1, and RNF8, and their respective WT counterparts were treated with HU, and immunostaining experiments were performed with anti-TopBP1 and anti-γH2AX antibodies. (D) Cells deficient in H2AX, MDC1, and their respective WT counterparts were treated with 3 μM aphidicolin for 16 h, and immunostaining experiments were performed with anti-TopBP1 and anti-RPA2 antibodies. (E) Extracts prepared from 293T cells expressing HA-tagged MDC1 were mock-treated or treated with 2-dimethyl-amino-4,5,6,7-tetrabromo-1H-benzimidazole (DMAT). Extracts were then incubated with bacterially expressed and purified GST or GST-BRCT4+5 fusion protein immobilized on glutathione agarose beads for 2 h at 4°C. The complex was subjected to Western blotting using the indicated antibodies. (F) HeLa cells were transfected with FLAG-tagged siRNA-resistant WT, D3 mutant, or 12A mutant of MDC1, and with MDC1 siRNA twice within a 24-h interval. Cells were selected with puromycin for 48 h and then treated with 10 mM HU for 16 h. Immunostaining experiments were performed with the indicated antibodies. The percentages of cells stained positive for 53BP1 foci were determined. Data are presented as mean ± SD (error bars) from three different experiments. Bars, 10 μm.

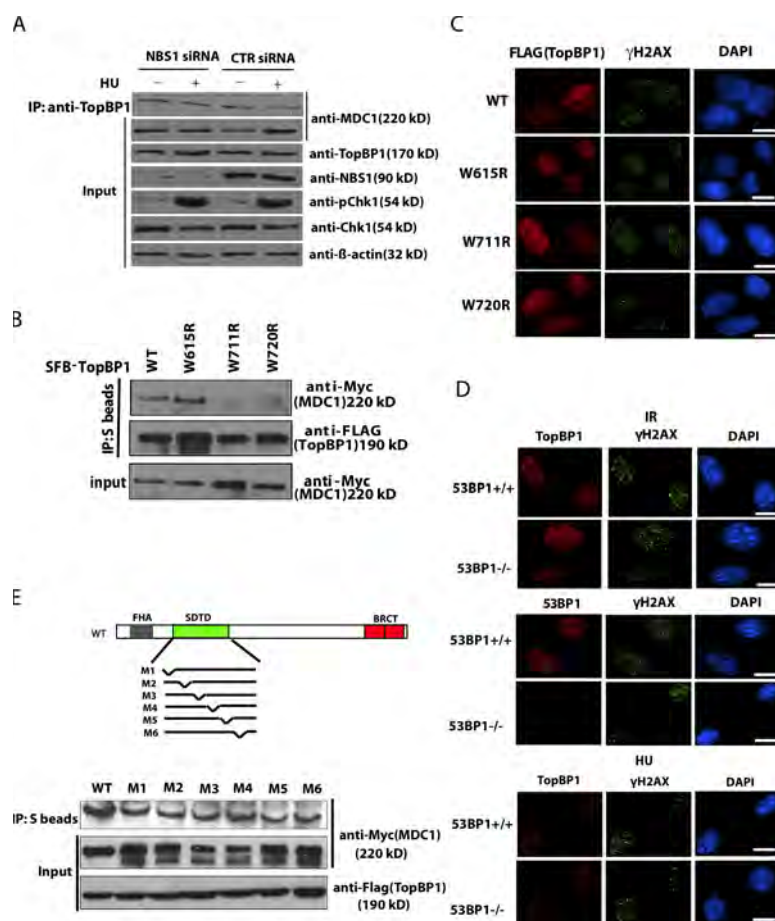


Figure S3. The phosphorylation-dependent interaction between MDC1 and TopBP1. (A) 293T cells were transfected with plasmids encoding Myc-tagged MDC1 together with plasmids encoding SFB-tagged WT or mutants of TopBP1. IP reactions were conducted using S protein beads, and then subjected to Western blotting using the indicated antibodies. (B) U2OS cells transfected with plasmids encoding SFB-tagged WT or mutants of TopBP1 were exposed to 2 mM HU for 2 h. Cells were fixed and immunostained with anti-FLAG and anti-γH2AX antibodies. (C) 53BP1^{-/-} and WT control cells were treated with HU or IR. Immunostaining was performed with the indicated antibodies. Bars, 10 μm. (D) Cells transfected with control or NBS1-specific siRNA were treated with or without HU for 1 h, and the interactions between TopBP1 and MDC1 were examined by a co-IP assay. Whole-cell extracts were immunoblotted with antibodies as indicated. (E) 293T cells were transfected with plasmids encoding SFB-tagged TopBP1 together with plasmids encoding WT or six small internal-deletion mutants (deleted residues 200–240, 240–280, 280–320, 320–360, 360–400, 400–440) of Myc-tagged MDC1. IP reactions were conducted using S protein beads and then subjected to Western blotting using the indicated antibodies.

The HARP domain dictates the annealing helicase activity of HARP/SMARCAL1

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Mutations in HepA-related protein (HARP, or SMARCAL1) cause Schimke immunoosseous dysplasia (SIOD). HARP has ATP-dependent annealing helicase activity, which helps to stabilize stalled replication forks and facilitate DNA repair during replication. Here, we show that the conserved tandem HARP (2HP) domain dictates this annealing helicase activity. Furthermore, chimeric proteins generated by fusing the 2HP domain of HARP with the SNF2 domain of BRG1 or HELLS show annealing helicase activity *in vitro* and, when targeted to replication forks, mimic the functions of HARP *in vivo*. We propose that the HARP domain endows HARP with this ATP-driven annealing helicase activity.

Keywords: annealing helicase; HARP; replication; RPA; Schimke immunoosseous dysplasia

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INTRODUCTION

Mutations in HepA-related protein (HARP); also known as DNA-dependent ATPase A and SMARCAL1 (SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily a-like 1) cause Schimke immunoosseous dysplasia (SIOD), a rare, autosomal recessive disease characterized by spondyloepiphyseal dysplasia, T-cell deficiency and focal segmental glomerulosclerosis (Boerkoel *et al*, 2002; Deguchi *et al*, 2008). HARP preferentially binds to forked DNA structures as opposed to single-stranded DNA (ssDNA) or double-stranded DNA (dsDNA), and the ATPase activity of HARP is stimulated by forked DNA structures (Muthuswami *et al*, 2000; Yusufzai & Kadonaga, 2008). Moreover, HARP possesses ATP-driven annealing helicase activity *in vitro* and can rewind complementary ssDNA bound by replication protein A (RPA; Yusufzai & Kadonaga, 2008). Recently, several groups including ours have proposed that the annealing helicase activity of HARP might dictate its role in S-phase-specific DNA damage response and that HARP is involved in protecting stalled replication forks by minimizing

the accumulation of ssDNA regions and facilitating the repair of collapsed replication forks during DNA replication (Yusufzai & Kadonaga, 2008; Bansbach *et al*, 2009; Ciccina *et al*, 2009; Postow *et al*, 2009; Yuan *et al*, 2009; Yusufzai *et al*, 2009).

HARP is a member of the conserved SNF2 family of proteins that are involved in chromatin remodelling and is required for transcriptional regulation, replication, recombination and DNA repair (Coleman *et al*, 2000). All members of the SNF2 family are characterized by the presence of the SWI/SNF helicase domain (SNF2 domain), but do not always exhibit helicase activity. Studies have shown that some of these proteins use the energy of ATP hydrolysis to translocate along the DNA and thus remodel chromatin by restructuring nucleosomes (Bork & Koonin, 1993; Eisen *et al*, 1995; Ristic *et al*, 2001; Saha *et al*, 2002; Durr *et al*, 2005; Fan *et al*, 2005).

HARP is an ATP-driven annealing helicase. The physiological relevance of HARP's annealing helicase activity was revealed by the finding that several mutations observed in SIOD patients result in mutant HARP proteins that have defective ATPase and annealing helicase activity, suggesting that the annealing helicase activity of HARP is important for its function *in vivo* (Boerkoel *et al*, 2002; Clewing *et al*, 2007; Yusufzai & Kadonaga, 2008). This speculation is supported by recent findings indicating that the S-phase functions of HARP also require its ATPase and annealing helicase activity (Bansbach *et al*, 2009; Ciccina *et al*, 2009; Postow *et al*, 2009; Yuan *et al*, 2009; Yusufzai *et al*, 2009). As all SNF2 family members have ATPase activity, we are interested in what determines the annealing helicase activity of HARP. In this study, we show that the conserved tandem HARP (2HP) domain of HARP protein dictates its annealing helicase activity. This activity is transferrable because when the SNF2 domain of Brahma-related gene 1 (BRG1); also known as SMARCA4) or helicase, lymphoid specific (HELLS); also known as SMARCA6, two other members of the SNF2 gene family is fused with the 2HP domain of HARP, the chimeric proteins have annealing helicase activity *in vitro*. Furthermore, the chimeric proteins are fully functional *in vivo* as they complement the function of HARP in HARP-depleted cells.

RESULTS AND DISCUSSION

HARP domain is required for HARP function *in vivo*

To gain a better understanding of the annealing helicase activity of HARP, we aligned the human HARP sequence with its orthologues

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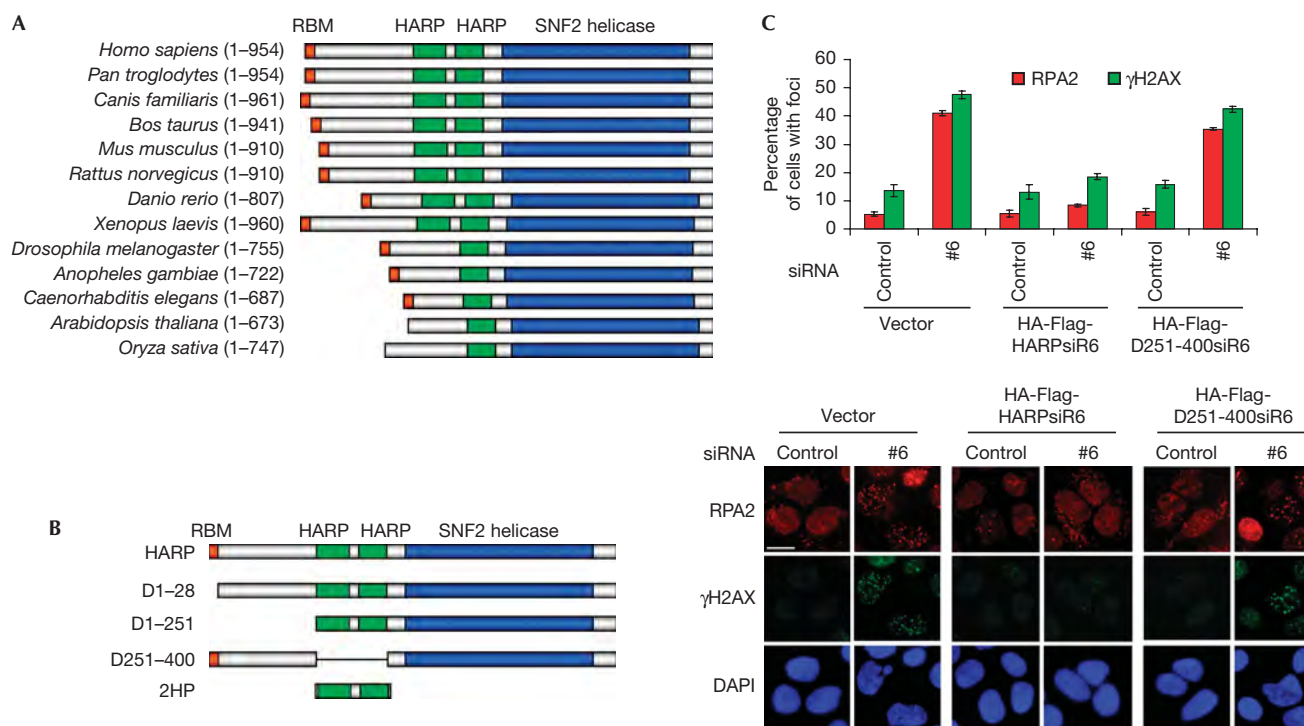


Fig 1 | The evolutionarily conserved HARP domain is crucial for HARP function. (A) All HARP orthologues contain the HARP domain and the SNF2 domain. (B) Schematic representation of wild-type HARP and the mutants used in this study. (C) The mutant lacking the 2HP domain could not restore HARP function *in vivo*. U2OS-derivative cells stably expressing siRNA-resistant wild-type HARP (HA-Flag-HARPSiR6) or the mutant lacking the 2HP domain (HA-Flag-D251-400siR6) were transfected with the indicated siRNAs; 72 h later, cells were subjected to immunostaining using RPA2 and γ H2AX antibodies as indicated. Foci-positive cells were quantified by counting a total of 200 cells per sample. Data are presented as mean \pm s.d. from three independent experiments. Scale bar, 10 μ m. DAPI, 4',6-diamidino-2-phenylindole; HA, haemagglutinin; HARP, HepA-related protein; RBM, RPA-binding motif; RPA, replication protein A; siRNA, short interfering RNA.

from other species. HARPs consist of approximately 700–950 amino acids and have three highly conserved regions: the amino-terminal RPA-binding motif (RBM; not found in *Arabidopsis thaliana* or *Oryza sativa*), the central single HARP (HP) or 2HP domain (approximately 60 residues each) and the carboxy-terminal conserved SNF2 domain (approximately 400 residues; Fig 1A).

Many mutations identified in SIOD patients are mapped to the conserved 2HP and SNF2 domains (Boerkoel *et al*, 2002; Clewing *et al*, 2007), suggesting that both domains are involved in the pathogenesis of SIOD. On the basis of these conserved domains and the mutations or deletions identified in SIOD patients, we generated a series of internal-deletion and truncation mutants of HARP (HARP D1–28, D1–251, D251–400 and 2HP; Fig 1B) and examined whether these mutants would affect the *in vivo* and *in vitro* functions of HARP. Studies have shown that the N-terminal RBM and the SNF2 domain of HARP are involved in stabilizing replication forks and that HARP-depleted cells display longer stretches of ssDNA during DNA replication, leading to collapsed replication forks and accumulation of dsDNA breaks (Bansbach *et al*, 2009; Ciccio *et al*, 2009; Postow *et al*, 2009; Yuan *et al*, 2009; Yusufzai *et al*, 2009). The appearance of RPA foci can be used as a surrogate marker for the accumulation of ssDNA regions in the cell (Zou & Elledge, 2003; Jazayeri *et al*, 2006). The frequency of dsDNA breaks can be monitored by the formation of phosphorylated H2AX (γ H2AX) foci. After endogenous HARP was

depleted, formation of RPA and γ H2AX foci was enhanced (Fig 1C). Interestingly, the expression of siRNA-resistant wild-type HARP, but not of the D251–400 mutant, which lacks the 2HP domain, suppressed the formation of RPA and γ H2AX foci. As a control, we show that the expression of D251–400 mutant is similar to that of wild-type HARP (supplementary Fig S1A online). This result and our previous data suggest that all three conserved domains of HARP are required for its functions *in vivo*.

HARP domain determines the annealing helicase activity

We asked whether the enhanced formation of RPA and γ H2AX foci observed in cells expressing the HARP D251–400 mutant (lacking the 2HP domain) was due to compromised DNA binding, DNA-dependent ATPase or the annealing helicase activity of this mutant. Both glutathione-S-transferase (GST)-tagged full-length HARP and its internal-deletion mutant proteins (Fig 2A) displayed DNA-binding activity with fork DNA (Fig 2B). The D1–28 and D1–251 mutants showed a modest reduction in DNA-binding activity, which could be due to the different oligomeric states of wild-type and mutant HARP proteins. However, size-exclusion chromatography of the wild-type and D1–28 mutant of HARP shows that both these proteins exist as monomers in solution (supplementary Fig S1B online). Consistent with earlier studies (Muthuswami *et al*, 2000; Yusufzai & Kadonaga, 2008), we found that fork DNA stimulates the ATPase activity of full-length HARP

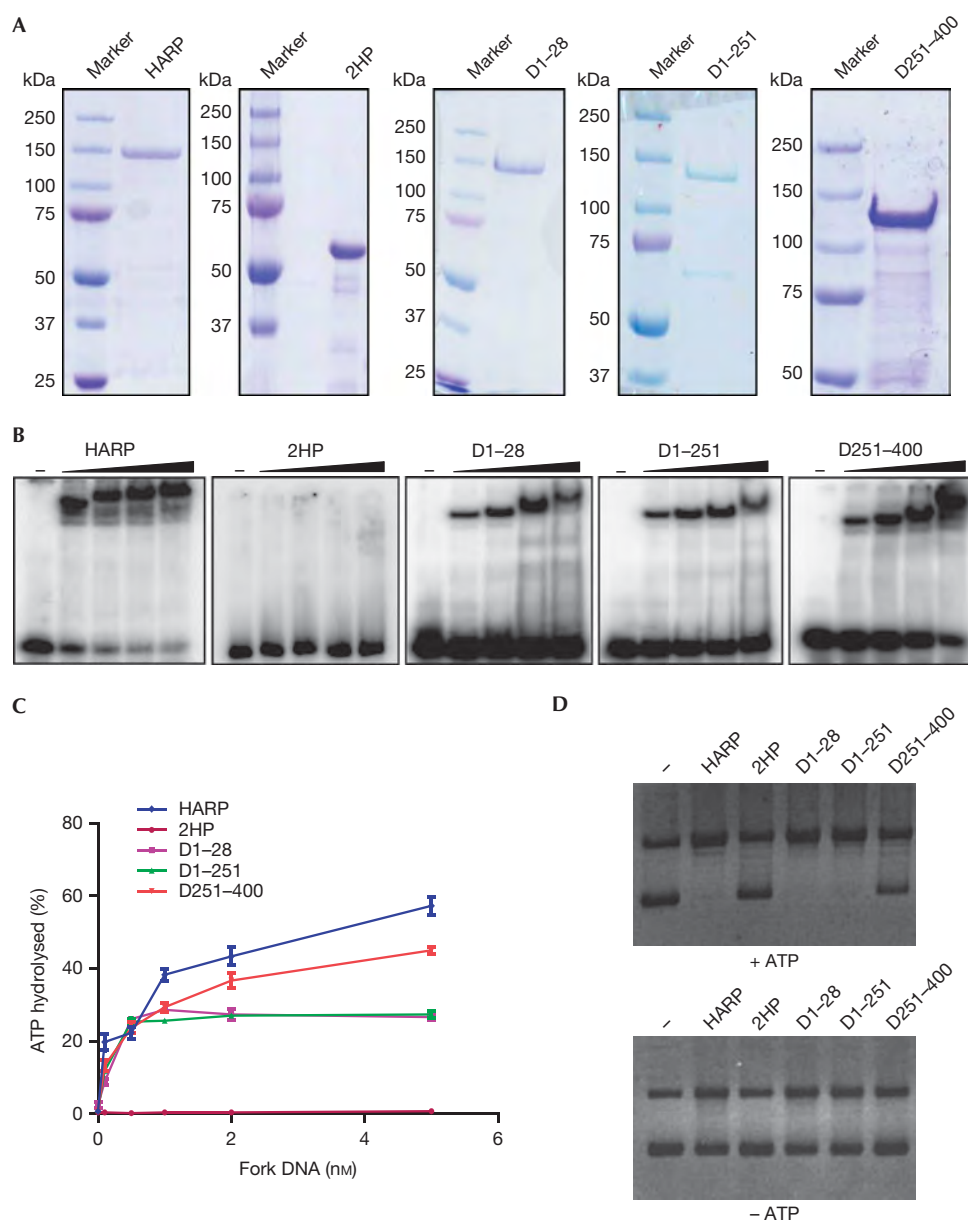


Fig 2 | The 2HP domain dictates the annealing helicase activity of HARP. (A) SDS-PAGE profile of purified wild-type and mutant HARP proteins. The indicated purified wild-type HARP and its internal-deletion mutants were analysed by 10% SDS-PAGE. (B) Wild-type and mutant HARP proteins bind to fork DNA. Fork DNA was incubated with increasing concentrations of the indicated wild-type and mutant HARP proteins, and reaction products were analysed by 8% native PAGE. (C) ATPase activity of wild-type and mutant HARP is stimulated by fork DNA. The indicated wild-type and mutant HARP proteins hydrolysed increasing amounts of ATP with increasing concentrations of fork DNA. The graph shows the percentage of ATP hydrolysed against the concentration of fork DNA. Data are plotted as mean \pm s.d. from three independent experiments. (D) Deleting the 2HP domain abolishes the annealing helicase activity of HARP. The annealing helicase assay was carried out with 100 nM HARP, 2HP, D1-28, D1-251 and D251-400 in the presence (top panel) or absence (bottom panel) of ATP. UTP was used as control in the absence of ATP. All reactions contained DNA, RPA and topoisomerase I. HARP, HepA-related protein; PAGE, polyacrylamide gel electrophoresis; RPA, replication protein A.

and its deletion mutants (Fig 2C). The observed reduction in the ATPase activity of the D1-28 and D1-251 mutants could be attributed to the reduced DNA-binding activity of these mutants (Fig 2B). The 2HP domain alone did not show DNA-binding or ATPase activity (Fig 2B,C). The 2HP domain also does not interact

with the RPA:DNA complex or the SNF2 domain of HARP (supplementary Figs S1C–E, S2A online).

Next, we examined the annealing helicase activity of wild-type and mutant HARP using a previously described assay (Yusufzai & Kadonaga, 2008). A partly unwound DNA substrate

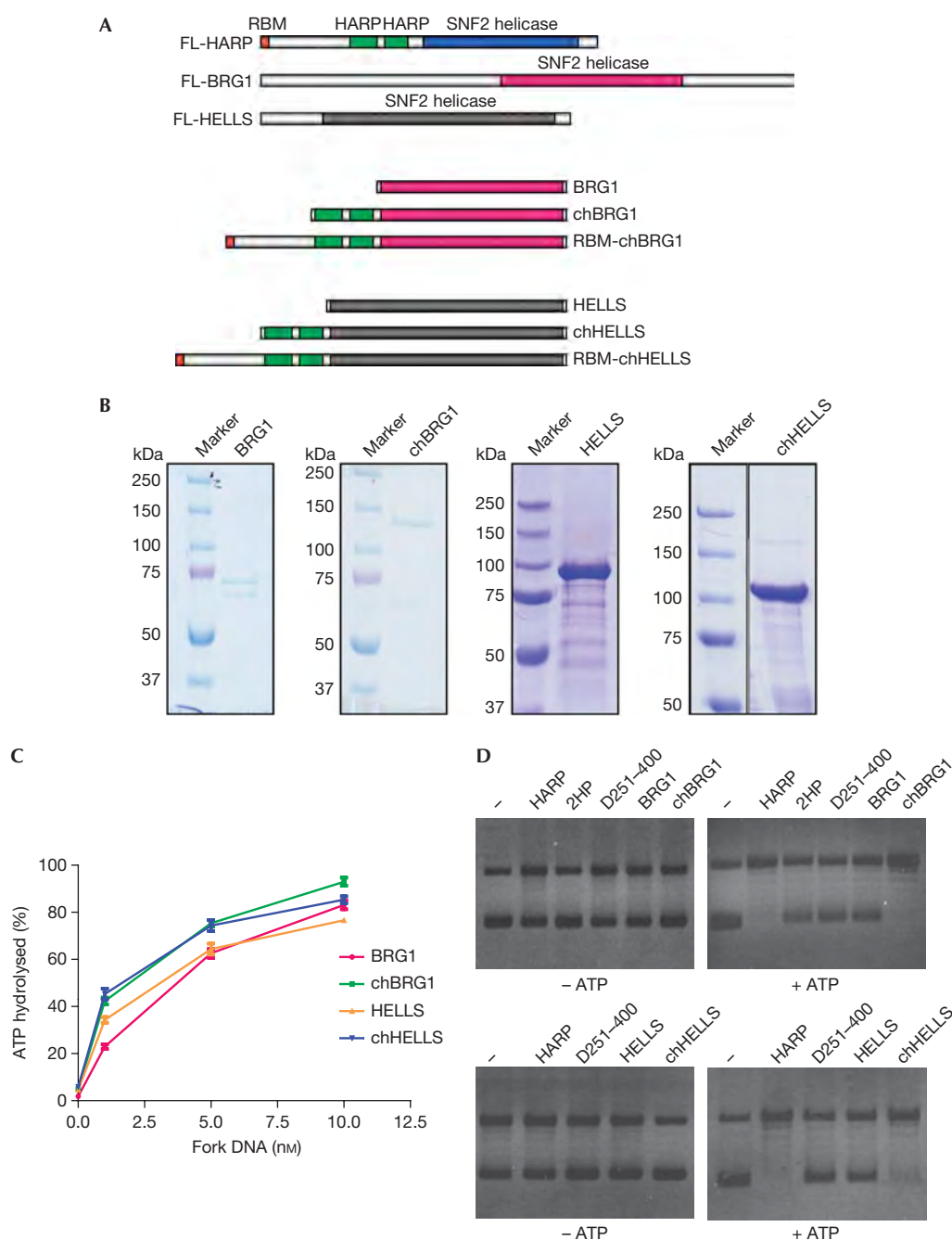


Fig 3 | Chimeric proteins have annealing helicase activity similar to that of wild-type HARP. (A) Schematic representation of chimeric constructs used in this study. (B) SDS-PAGE profile of purified chimeric proteins. GST-tagged BRG1, chBRG1, HELLS and chHELLS were purified from Sf9 cells and analysed by 10% SDS-PAGE. (C) BRG1, chBRG1, HELLS and chHELLS all display DNA-dependent ATPase activity. The graph shows the percentage of ATP hydrolysed against the concentration of fork DNA. Data are plotted as mean \pm s.d. from three independent experiments. (D) chBRG1 and chHELLS have annealing helicase activity. The annealing helicase assay was carried out using the indicated purified proteins in the absence (left panels) or presence (right panels) of ATP. UTP was used as a control in the absence of ATP. All reactions contained DNA, RPA and topoisomerase I. BRG1, Brahma-related gene 1; HARP, HepA-related protein; HELLS, helicase, lymphoid specific; PAGE, polyacrylamide gel electrophoresis; RBM, RPA-binding motif; RPA, replication protein A.

was generated by treating plasmid DNA with RPA in the presence of topoisomerase I. Addition of wild-type HARP, D1–28 and D1–251 mutants catalysed the annealing of RPA-unwound DNA only in the presence of ATP (Fig 2D). However, the D251–400 mutant

of HARP failed to do so (Fig 2D). Furthermore, two other HARP internal deletion mutants (D288–366 and D366–383) identified in SIOD patients (Clewning *et al*, 2007) that contain a partial or an intact HP domain failed to function as an annealing helicase

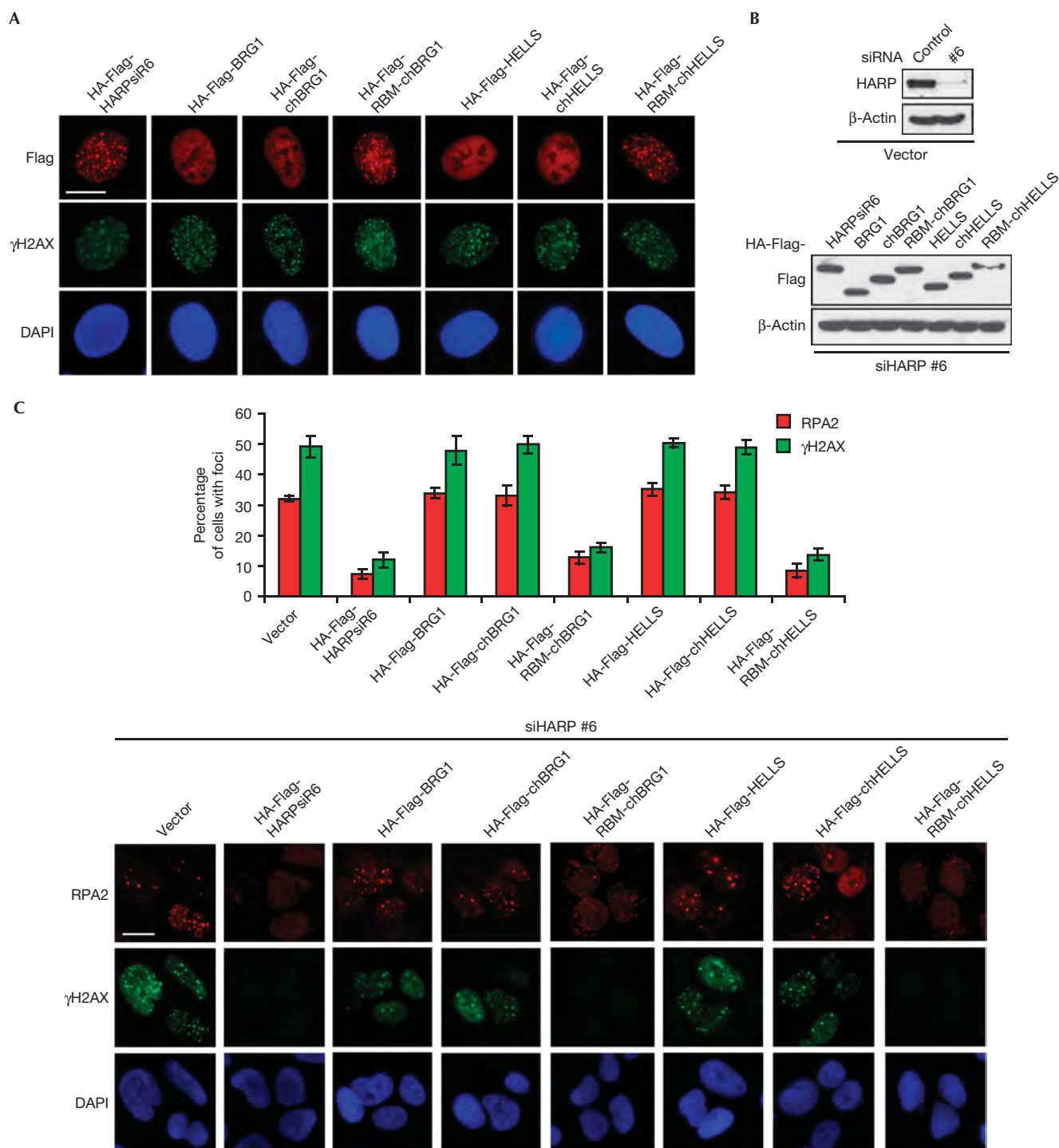


Fig 4 | Chimeric proteins containing RBM can restore HARP function *in vivo*. (A) U2OS-derivative cells stably expressing HA-Flag-tagged fusion proteins or the SNF2 domain alone (BRG1 and HELLS), chimeras lacking RBM (chBRG1 and chHELLS), and chimeras with RBM (RBM-chBRG1 and RBM-chHELLS) were generated. The hydroxyurea-induced foci-forming abilities of these fusion proteins were determined by immunostaining using the indicated antibodies. (B) The endogenous and exogenous expression levels of HARP and the chimeric proteins were confirmed by immunoblotting using the indicated antibodies. The extracts were prepared from cells transfected with indicated siRNAs. (C) U2OS-derivative cells were transfected with the indicated siRNAs; 72 h later, cells were subjected to immunostaining using RPA2 and γ H2AX antibodies. Foci-positive cells were quantified by counting a total of 200 cells per sample. Data are presented as mean \pm s.d. from three independent experiments. Scale bars, 10 μ m. BRG1, Brahma-related gene 1; DAPI, 4',6-diamidino-2-phenylindole; HA, haemagglutinin; HARP, HepA-related protein; HELLS, helicase, lymphoid specific; RBM, RPA-binding motif; RPA, replication protein A; siRNA, short interfering RNA.

in vitro (supplementary Fig S2B online), suggesting that the tandem HP domains are required to direct the annealing helicase activity of human HARP. Together with the *in vivo* study (Fig 1C), these data imply that the conserved 2HP domain is not involved in DNA-binding or ATPase activity, but determines the annealing helicase activity of HARP *in vitro* and is required for its function in stabilizing replication forks *in vivo*.

Chimeric proteins display annealing helicase activity

As the 2HP domain alone lacks DNA-binding, ATPase or annealing helicase activity (Fig 2), we suggest that this domain has to function together with the SNF2 domain of HARP to carry out its annealing helicase activity. We asked whether the SNF2 domain of HARP has unique features or whether the 2HP domain of HARP can be transferred to other SNF2 family proteins. We generated chimeric proteins by fusing the 2HP domain with other proteins that belong to the SNF2 family. BRG1 is the central catalytic component of several multi-subunit chromatin-remodelling complexes (Reisman *et al*, 2009). Similarly to other members of the SNF2 family, BRG1 harbours the conserved SNF2 domain, but lacks the 2HP domain (Fig 3A) and does not have annealing helicase activity (Yusufzai & Kadonaga, 2008). We created a chimera, chBRG1, in which the 2HP domain of HARP was fused with the SNF2 domain of BRG1 (Fig 3A). GST-fused SNF2 domain of BRG1 and GST-chimeric BRG1 (GST-chBRG1; Fig 3B) both displayed DNA-dependent ATPase activity (Fig 3C). Although BRG1 does not have annealing helicase activity, the chimeric protein chBRG1 showed annealing helicase activity comparable to that of HARP (Fig 3D).

To confirm these results we generated another chimera, chHELLS (Fig 3A), in which the 2HP domain of HARP was fused with the SNF2 domain of HELLS, a protein that has a role in cellular proliferation and leukaemogenesis (Raabe *et al*, 2001). Again, both HELLS and chHELLS displayed DNA-dependent ATPase activity (Fig 3C). However, chHELLS, but not HELLS, exhibited annealing helicase activity similar to that of full-length HARP (Fig 3D; supplementary Fig S1F online). Furthermore, we detected that HARP and the chimeric proteins exhibited annealing helicase activity on DNA coated with *Escherichia coli* single-strand DNA-binding protein (SSB), indicating that HARP or these chimeric proteins have general annealing helicase activity that does not depend on or require any specific SSBs (supplementary Fig S2C online). Together, these results suggest that the 2HP domain of HARP protein determines the annealing helicase activity of HARP. It functions together with the SNF2 domain, which is involved in ATP binding and hydrolysis, and promotes the annealing of complementary ssDNA strands.

Chimeric proteins carry out HARP function *in vivo*

We wondered whether the chimeric proteins could be substituted for HARP *in vivo* if, similarly to HARP, they had annealing helicase activity *in vitro*. HARP is recruited to stalled replication forks by means of its direct interaction with RPA, although RPA-binding activity is not required for its annealing helicase activity *in vitro* (Bansbach *et al*, 2009; Ciccina *et al*, 2009; Yuan *et al*, 2009; Yusufzai *et al*, 2009). To ensure that the chimeric proteins were correctly localized in the cell, we generated siRNA-resistant fusion constructs (RBM-chBRG1 and RBM-chHELLS) in

which RBM was fused to the chimeric proteins (Fig 3A). The mutants with RBM, similarly to wild-type HARP, formed nuclear foci after hydroxyurea treatment (Fig 4A). The expression of these mutants and the knockdown of endogenous HARP in U2OS cells were confirmed by western blot analysis (Fig 4B). Expression of siRNA-resistant RBM-chBRG1 and RBM-chHELLS suppressed the enhanced formation of RPA and γ H2AX foci after endogenous HARP was depleted (Fig 4C). However, the SNF2 domain alone (BRG1 and HELLS) or the chimeras lacking RBM (chBRG1 and chHELLS) failed to restore the function of HARP *in vivo* (Fig 4C). Moreover, we generated two fusion proteins, RBM-BRG1 and RBM-HELLS, without 2HP domain. Although these fusion proteins formed nuclear foci after hydroxyurea treatment, they could not restore HARP function *in vivo* (supplementary Fig S3 online). In addition, RBM-chBRG1 and RBM-chHELLS, purified from mammalian cells, had annealing helicase activity similar to that of HARP *in vitro* (supplementary Fig S2D online). Again, these data suggest that the 2HP domain is required and dictates the annealing helicase activity and function of HARP protein.

Stalled replication forks might arise during normal chromosome replication or in the presence of DNA lesions (Walter & Newport, 2000; Tercero & Diffley, 2001; Katou *et al*, 2003; Pacek *et al*, 2006). These stalled replication forks, if they are left unrepaired, are unstable because of the presence of long stretches of ssDNA. They can collapse into deleterious structures that prevent resumption of DNA replication and lead to unscheduled recombination, resulting in cell death or genomic instability. Although the mechanisms involved in protecting stalled replication forks are unknown, it has been speculated that the annealing helicase activity of HARP might have a role in either stabilizing stalled forks or mediating the repair of collapsed replication forks, as it can rewind RPA-coated ssDNA regions to form more stable duplex DNA (Yusufzai & Kadonaga, 2008; Bansbach *et al*, 2009; Ciccina *et al*, 2009; Postow *et al*, 2009; Yuan *et al*, 2009; Yusufzai *et al*, 2009). In this study, we demonstrate that the evolutionarily conserved HARP domain determines the annealing helicase activity required for the *in vivo* and *in vitro* functions of HARP. Moreover, the HARP domain is a distinct functional domain because it can be transferred to other SNF2 family members. Structural analysis is needed to uncover the properties of the HARP domain and the catalytic residues responsible for the annealing helicase activity. Another protein with annealing helicase activity, annealing helicase 2 (AH2), previously termed ZRANB3 (zinc-finger, RAN-binding domain containing 3), has been recently identified (Yusufzai & Kadonaga, 2010), implying that there might be a subset of the SNF2 family of proteins that process annealing helicase activity. Similarly to HARP, AH2 displays DNA-dependent ATPase activity and catalyses ATP-dependent rewinding of RPA-coated ssDNA. AH2 lacks the N-terminal RBM found in HARP, but harbours a conserved SNF2 domain and HNH motif (Yusufzai & Kadonaga, 2010). Alignment of HARP domains with AH2 reveals a putative 'HARP-like' domain in the AH2 protein (residues 712–820; supplementary Fig S4 online). This 'HARP-like' domain has several residues that are conserved in all of the HARP proteins (data not shown). It would be interesting to test whether this putative 'HARP-like' domain is crucial for the function of AH2 as an annealing helicase.

METHODS

Plasmid constructs, antibodies, cell culture, transfection and siRNAs, DNA substrates protein purification in insect cells, electrophoretic mobility shift assay and the ATPase assay used in this study are described in the supplementary information online.

Immunostaining. Cells cultured on coverslips were washed with PBS, pre-extracted with 0.5% Triton X-100 solution for 3 min and fixed with 3% paraformaldehyde for 12 min. Coverslips were washed with PBS and then immunostained with primary antibodies in 5% goat serum for 60 min. Coverslips were then washed and incubated with secondary antibodies conjugated to rhodamine or fluorescein isothiocyanate for 60 min. Cells were then stained with 4',6-diamidino-2-phenylindole (DAPI) to visualize nuclear DNA. The coverslips were mounted onto glass slides with antifade solution and visualized using a Nikon Eclipse E800 fluorescence microscope with a Nikon Plan Fluor $\times 40$ oil objective lens (NA 1.30) at 27 °C. Cells were photographed using a SPOT camera (Diagnostic Instruments) and analysed using Photoshop software (Adobe).

Annealing helicase assay. The annealing helicase assay was carried out as described previously (Yusufzai & Kadonaga, 2008), with the following modifications. Supercoiled pUC19 DNA (0.2 μ g) was incubated with 2.5 μ g of purified RPA or *E. coli* SSB (Episentre Biotechnologies; supplementary Fig S2C online) in the presence of 40 mM Tris-HCl (pH 8.0), 20 mM NaCl, 1 mM EDTA, 5 mM MgCl₂ and 5 mM dithiothreitol at 37 °C for 45 min. The reaction mixture was then treated with 2 units of *E. coli* topoisomerase I (New England BioLabs) and incubated at 37 °C for another 30 min. The indicated proteins (at 100 nM) were added, and the mixture was incubated for a further 30 min at 37 °C. The reaction was terminated with a solution of SDS-EDTA. The products were extracted with an equal volume of chloroform and resolved on 1.2% agarose gel at 40 V/cm for 5 h. The gel was then stained with ethidium bromide.

Supplementary information is available at EMBO reports online (<http://www.emboreports.org>).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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